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BULLETIN OF THE RESEARCH COUNCIL OF ISRAEL

Section A

CHEMISTRY

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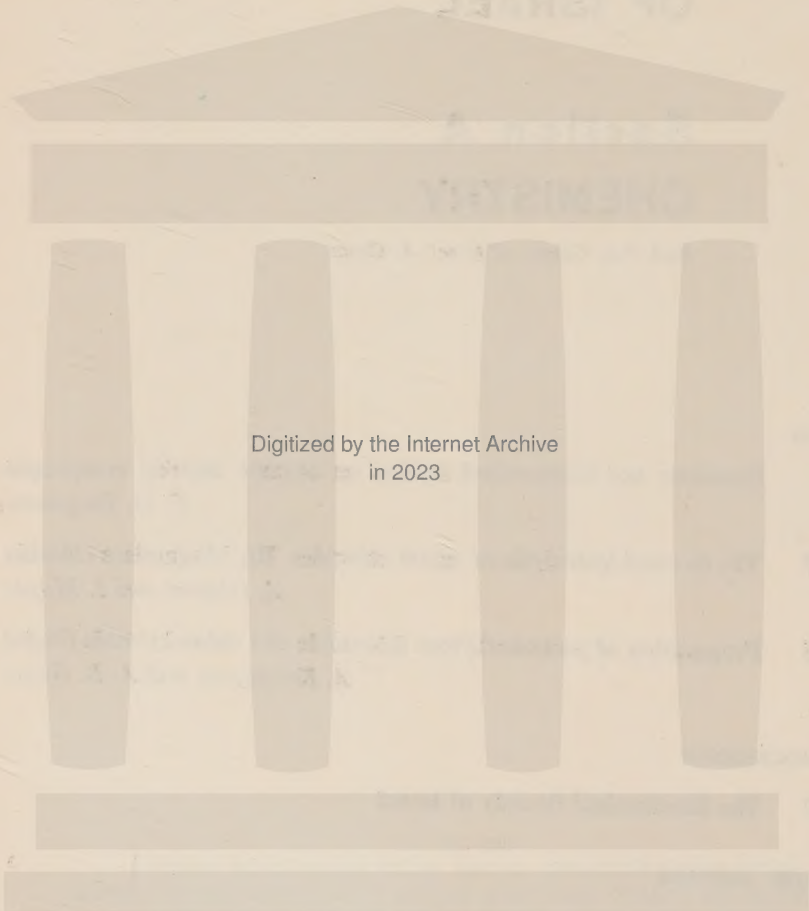

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• Volume 10A, Number 1, April 1961

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SYNTHETIC AND BIOCHEMICAL STUDIES ON ORGANIC FLUORINE COMPOUNDS*

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Department of Organic Chemistry, The Hebrew University of Jerusalem

The aromatic fluorine compounds have been known for some time, as far as their preparation and chemical properties are concerned. The reaction of diazonium salt solutions with hydrofluoric acid leads in many cases to aromatic fluorides; but the now classical method of the thermal decomposition of the relatively water-insoluble and stable diazonium fluoroborate is the method of choice and gives — generally speaking — satisfactory results. In some cases, variations have been found useful. It has, e.g., been found that in certain instances the diazonium fluoroborates are water-soluble, e.g. those of monoacylarylenediamines; in these cases, the aqueous solutions can be transformed into fluorine compounds by catalytic influence of copper powder at ordinary temperature. [1,2]. This method has been extended to a decomposition of the fluoroborates, dissolved in boiling aqueous acetone, in the presence of cuprous chloride or cupric fluoride (catalytic amounts)[3]; it is applicable when the molecule does not contain substituents other than alkyl, aryl or halogen[4]. The procedure suggests a radical mechanism, as, indeed, it has been discussed for such decomposition reactions;[5] this hypothesis has been strengthened by the observation that optically active 2,2′-diamino-6,6′-dimethylbiphenyl gives an optically active diazonium salt, but — under the conditions defined — a practically racemic 2,2′-difluoro-6,6′-dimethylbiphenyl.[4] A mechanism involving either negative or positive *ions* would — for electrostatic reasons — presumably not have led to racemization.

However, the most interesting property of aromatic fluorine compounds is the fact that aromatic metabolites are transformed by fluorine substitution into anti-metabolites. *p*-Fluorophenylalanine and the *o*-isomer, for example, are toxic for cells which require phenylalanine,[6,7] 5- and 6-fluorotryptophan play the same role for trytophan-requiring organisms, [8, 8a] and 3-fluoro-4-aminobenzoic acid is an antagonist to *p*-amino-benzoic acid.[9] This toxicity is of the competitive type, reversed by an excess of the respective metabolite. This statement is, however, incomplete. One knows that the fluorinated phenylalanines are incorporated into cell protein instead of the fluorine-free parent substance, [10,11] and the fact that the fluorinated tryptophans are substrates for the trytophan-activating enzyme, as

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* Lecture delivered at the spring 1960 meeting of the Israel Chemical Society

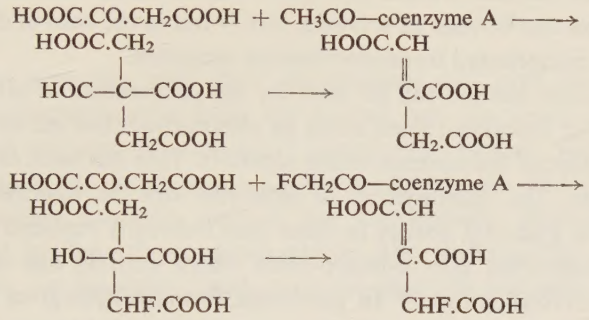
Received September 7, 1960.

Sharon[8] Lipman and have found, must probably be interpreted analogously. This is even more striking, if one considers that *p*-chlorophenylalanine which *electronically* resembles the fluorine compound fairly closely, is not incorporated into protein,[6,7] nor is this the case for 5- or 6-methyltryptophan,[6,7,12] although the molecular *weight* of the methyl group is practically identical with the atomic weight of the fluorine atom. It is, therefore, neither the weight nor the electrochemical character which gives these fluorine compounds their peculiar biological properties. It is the radius which is so near that of the hydrogen, that the geometrical shape of the molecule is obviously not changed when an atom of hydrogen is exchanged by one of fluorine. The atomic radius of the hydrogen is 0.35, that of fluorine 0.64 Å, and if one — as one should — compares the van der Waals radii, the similarity is still more striking — 1.20 and 1.35 Å. This similarity expresses itself also in the fact, that in many compounds hydrogen can be replaced isomorphogeneously by fluorine[13].

It appears, therefore, that the cell cannot always discriminate between hydrogen and fluorine compounds; the fluorine compound is incorporated instead of the parent substance, and the fluorine compounds so formed can participate in further steps of the metabolism. One will realise that a number of possibilities exists as to the mode of action of these antimetabolites, even if one has not always succeeded in differentiating between them. The fluorine compound can be an inhibitor as such, for example, combine with an enzyme [13a] and prevent thus the normal reaction of the metabolite. It can be metabolised by this enzyme, serving as an alternative substrate, and in this way (or directly) be incorporated into cell constituents.[13b] This may be without influence on the cell, or the latter may — at some stage — "become aware" of the anomaly; the product formed from the initial fluorine compound will then be toxic, and we have before us a case of lethal synthesis, to use an expression coined by Peters.[14] Whatever the mechanism may be, the similarity in the size of the hydrogen and the fluorine atoms is of decisive importance, a fact which opens the way for many interesting speculations and experiments. To mention only one: the carcinogeneity of certain polycyclic hydrocarbons has been ascribed to a particular electron density in the so-called *K*-region of the molecule; it is also determined by the completely planar character of the molecule. The geometrical shape of the molecule will not be changed by the introduction of a fluorine into the *K*-region, the electron density will. What will be the carcinogeneity of the fluorine compounds? Experiments in this direction have been undertaken in several laboratories.[15–20]

In two African plants, *Dichapetalum cymosum* and *toxicarium*, fluoroacetic acid has been found; these plants are extremely poisonous to cattle (and human beings), and their toxicity is due to that simple compound — fluoroacetic acid. The chemist will ask at once two questions. Firstly: how is the C-F bond formed from inorganic fluoride and how does the plant collect the fluorine which is one of the minor trace elements? Secondly: why is fluoroacetic acid toxic? There is no answer yet to the first question; it may be, that a compound like the phosphoric acid ester of glycolic acid can exchange the phosphate radical for the fluoride ion; we have,

indeed, observed similar reactions. To the second question, an answer has been given by Peters, [14,21] who postulated that fluoroacetic acid enters, instead of acetic acid, the Krebs cycle and forms fluorocitrate by the well-known enzymatic reaction:



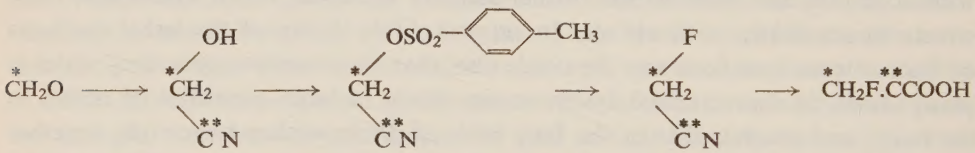
At this stage, however, the cycle is interrupted, fluorocitrate combines preferentially with aconitase and prevents the further steps of the cycle, which would lead from citrate to aconitate, isocitrate etc. In support of this theory of the lethal synthesis of fluorocitrate, two facts can be cited: one, that fluoroacetate poisoning which is always fatal, is characterized by an accumulation of large quantities of citrate in the body, and second, that in the fatty acids of *Dichapetalum toxicarium*, together with fluoroacetic acid, a fluoropalmitic acid occurs; its structure has not been ascertained unequivocally, but it is obvious that this acid can only be formed by a "mixed" fatty acid synthesis, using both acetate and fluoroacetate — in appropriate form — as substrates.

It seemed, therefore, worthwhile to synthesize the fluoro-analogs of the members of the Krebs cycle and to study their biological behaviour, and furthermore, to inquire whether fluoroacetic acid can also replace acetic acid in the other numerous systems which are based on this compound — the fatty acids, the carotenoids and the other isoprenoids, the steroids and the many other complicated natural products of greater attraction to the organic chemist because of their complexity, but of lower biological importance. We intended to prepare the fluoroanalogs of the intermediate steps of these biosynthetic pathways and to study their behaviour. As aliphatic fluorine compounds of the type of fluoroacetic acid were fairly difficult to obtain until now and to a large extent even unknown, we had to devote much of our efforts so far to the synthesis and the study of the properties of these simple fluoro-compounds. We expected by our efforts to open the route also to the fluorine analogs of other important members of the intermediate metabolism, the sugars, the amino-acids, the pyrimidines and purines etc.

Fluoroacetate has become very easily available and has been produced in our laboratories in kilogram quantities: one heats, in a column, a mixture of an appropriate chloroacetic acid ester with potassium fluoride in acetamide as solvent; the corresponding fluoroacetate, which has a much lower boiling point than the chloro-

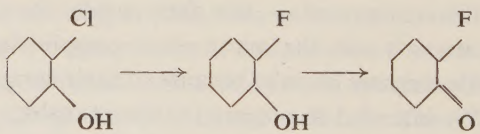
ester, distils off continuously. [22] Acetamide has one advantage compared with other solvents: it dissolves potassium fluoride at 100° to about 8%, and thus permits reaction in homogeneous phase. The potassium chloride formed, on the other hand, is insoluble and is thus eliminated from the equilibrium. In many cases, diethylene glycol and ethylene glycol can be used as solvents, too, [23] but in the case of chloroacetate they interfere due to complicated transesterification reactions.

This simple acetamide method can be used for the preparation of alkyl fluorides from alkyl chlorides or bromides; there exists an alternative in the use of the toluene- or methane-sulphonates of the corresponding alcohols. This has been of importance in one particular case. The cyanohydrins of aldehydes can be esterified with these sulfonic acids and the hydroxyl groups in them thus indirectly replaced by fluorine: the aldehydes are converted into α-fluoro-acids which contain one carbon atom more, that of the carboxyl group. [24] In particular, formaldehyde gives thus fluoroacetonitrile, in a reaction which permits the easy preparation of either or both methylene- and carboxyl-labeled fluoroacetic acid:



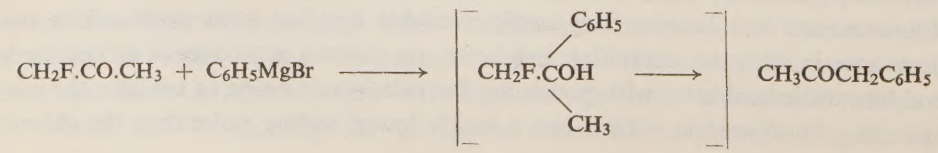
The method described for the transformation of chloro-into fluoro-compounds proved especially fruitful — for reasons not entirely clear — in the case of chloro-alcohols:

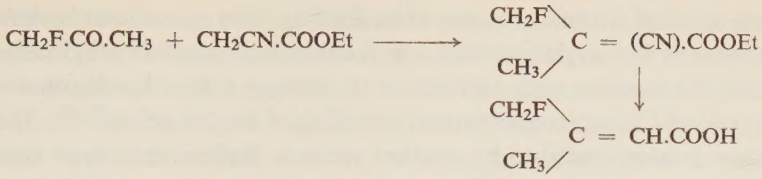
$$CH_2Cl—CH_2OH \longrightarrow CH_2F—CH_2OH$$
$$CH_2Cl.CHOH.CH_3 \longrightarrow CH_2F.CHOH.CH_3 \longrightarrow CH_2F.CO.CH_3$$
$$[CH_2Cl.CO.CH_3 \longrightarrow CH_2F.CO.CH_3]$$
$$CH_2Cl.CHOH.CH_2Cl \longrightarrow CH_2F.CHOH.CH_2F \longrightarrow CH_2F.CO.CH_2F$$
$$CH_2Cl.CHOH.CH_2OH \longrightarrow CH_2F.CHOH.CH_2OH \longrightarrow CH_2F.CHOH.COOH$$



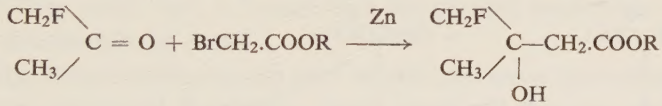
The fluoro-alcohols so obtained offer the easiest way to the preparation of α-fluoroketones, though in some cases the direct replacement of chlorine in α-chloroketones is possible. [25] From 1-fluoro-2,3-propanediol, β-fluorolactic acid has become readily accessible.[26]

The ketones, especially fluoroacetone and difluoroacetone, have been extensively studied:



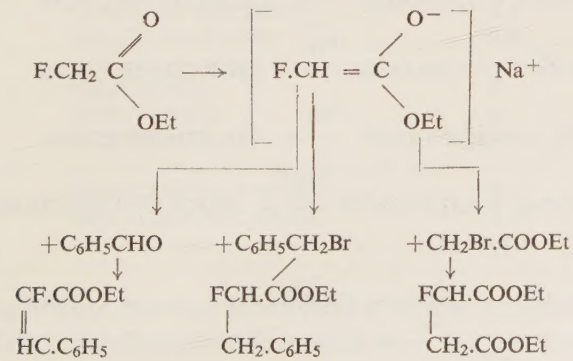


They react normally with ethyl cyanoacetate and give rise to the fluorinated α-cyano-β,β-dimethylacrylic acids which lead into the series of the intermediates of carotenoid and steroid synthesis.[27] Surprisingly enough, the usually very strong C-F bond does not stand up to Grignard compounds. Fluoroacetone gives with phenylmagnesium bromide, even at low temperature, phenylacetone, which can be explained by the scheme given above. The Reformatzky reaction with ethyl bromoacetate, on the other hand, and zinc proceeds normally: [27, 28]



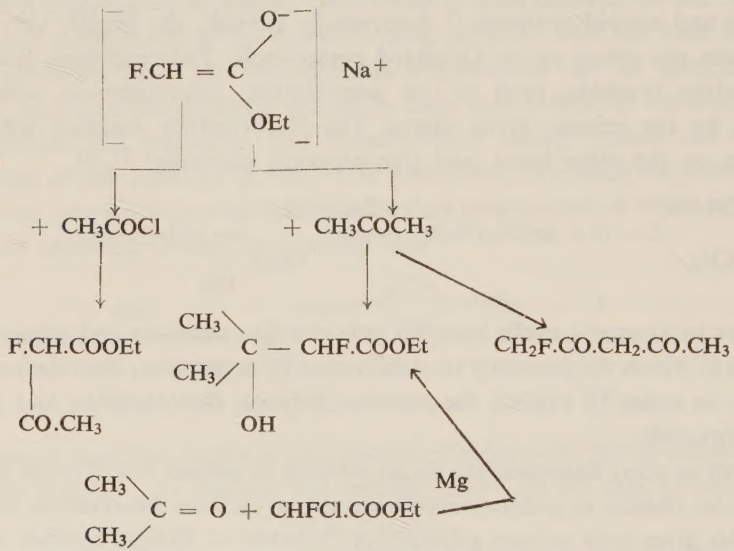
One will have to assume a cyclic complex between fluoroacetone and phenymagnesium bromide in which the tendency to stabilization by magnesium fluoride formation is very great, in order to explain the reaction between fluoroacetone and phenylmagnesium bromide.

Let us return to ethyl fluoroacetate. In an attempt to subject it to *Claisen* condensation, so as to obtain α, γ-difluoroacetoacetate, a curious observation has been made: the ester gives with sodium alkoxide or — better — sodium hydride a precipitate which seems rather inert and upon hydrolysis yields again ethyl fluoroacetate; only slowly is the substituted acetoacetate formed. [29] We have been able to prove that the precipitate is the fairly stable enolate of ethyl fluoroacetate. This has opened up many synthetic possibilities:



The Perkin condensation gives ethyl α-fluorocinnamate with benzaldehyde and can be performed with a large number of other aromatic aldehydes. [30] Alkylation

with reactive alkyl bromides is possible, leading here to α-fluorohydrocinnamate and -succinate.[29] The acylation with acetyl chloride [31] leads to ethyl α-fluoroacetoacetate and the reaction with acetone — in analogy with other ketones — to two products: one of Claisen condensation, one of aldol condensation[27,32]. The product of the latter is also available by another route, a Reformatzky-type condensation of acetone and the easily available ethyl chlorofluoroacetate, which, however, has to be carried out with magnesium and not with zinc: [26,28]



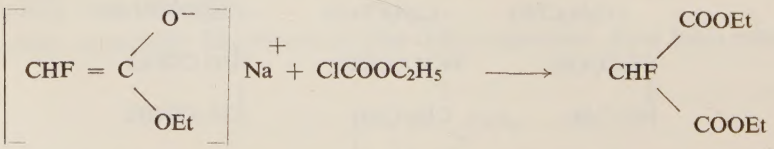
This observation has been useful in our attempts to prepare the reactive and therefore very interesting fluorinated β-keto-esters. [31]

$$CH_2F.COOEt + CH_2F.COOEt \xrightarrow{NaH} CH_2F.CO.CHF.COOEt$$

$$CH_2F.COOEt + BrCH_2COOEt \xrightarrow{Mg} CH_2F.CO.CH_2.COOEt$$

$$CH_3COOEt + CHClF.COOEt \xrightarrow{Mg} CH_3.CO.CHF.COOEt$$

$$COOEt.COOEt + CH_2F.COOEt \xrightarrow{NaH} EtOOC.CO.CHF.COOEt$$



We have seen already that self-condensation of ethyl fluoroacetate leads to ethyl α,γ-difluoroacetoacetate, [33,34] and that ethyl α-fluoroacetoacetate can be made

by acetylation of the enolate of fluoroacetate. [31] However, there is another possibility, equally applicable to the preparation of ethyl γ-fluoroacetoacetate: the condensation of chlorofluoroacetate with acetate and that of bromoacetate with fluoroacetate, both carried out with the help of magnesium. Though not without analogies, [35] these reactions are somewhat unusual, but they proceed smoothly and make the fluorinated acetoacetates easily available.

The important oxalofluoroacetate has been made from fluoroacetate and oxalate[36-38], and fluoromalonate by reaction of the enolate of fluorocetate with ethyl chloroformate, [39] or — recently — by oxidation of oxalofluoroacetate with Caro's acid.[40] It may be interesting to record that in spite of the description in the literature,[41] the reaction of bromomalonate or chloromalonate with potassium fluoride does not give fluoromalonate, although the product has a sharp boiling point and the correct analysis. It is a mixture of diethyl malonate and diethyl difluoromalonate which have the same boiling point, and is formed by a complicated mechanism which I would like not to discuss here in detail.

Incidentally, difluoromalonate can also be obtained from the enolate of malonate and the newly introduced fluorinating agent perchloryl fluoride, which substitutes all reactive methylene and methine groups by fluoride. [42] It is a most interesting compound, but very dangerous, and has led in our laboratory to a serious accident.

We have, thus, made available a series of ketones and keto esters which offer almost unlimited opportunities for further and more complicated syntheses. Of course, we have also studied the effect of fluorine substitution on the physical properties of the parent substances, such as the spectra; here, however, I would only like to give some measurements of the enol-ketone equilibrium.[43]

	% enol
$CH_3.CO.CH_2COOEt$	4.4
$CH_2F.CO.CH_2.COOEt$	18.2
$CH_3CO.CHF.COOEt$	0.99
$CH_2F.CO.CHF.COOEt$	5.2
$CF_3.CO.CH_2.COOEt$	49.0
$CH_2(COOEt)_2$	0
$CHBr(COOEt)_2$	0
$CHF(COOEt)_2$	0.91
$EtOOC.CO.CH_2.COOEt$	75
$EtOOC.CO.CHF.COOEt$	2.77

In the acetoacetate series, fluorine in α-position inhibits enolization, in γ-position increases it. Equally, oxalofluoroacetate is much more ketonic than the fluorine-free parent substance. It is interesting that fluoromalonate is slightly enolized, whilst malonate and bromomalonate are not.

I would rather turn to some chemical reactions of these compounds. The aceto-

xcetates can be reduced to the fluoro-analogs of the biologically important hydrorybutyric acid, and dehydration leads to fluorinated crotonic acids.[27]

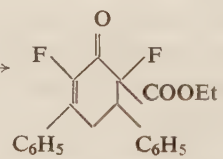
$$CH_2F.CO.CH_2.COOEt \xrightarrow{H_2} CH_2F.\underset{\displaystyle OH}{\underset{|}{CH}}.CH_2.COOEt \xrightarrow{-H_2O} CH_2F.CH = CH.COOEt$$

Both the fluoroacetoacetates and fluoromalonate are capable of Michael reactions;[44] the following chart shows some of them.

$$\begin{matrix} & COOEt & & & & COOEt \\ & \diagup & & & & \diagup \\ CHF & & + CH_2 = CH.CO.CH_3 & \longrightarrow & CH_3.CO.CH_2.CH_2.CF & \\ & \diagdown & & & & \diagdown \\ & COOEt & & & & COOEt \end{matrix}$$

$$CH_3COCHFCOOEt + CH_2 = CH.CN \longrightarrow CH_3.CO.\underset{\displaystyle CH_2.CH_2.CN}{\underset{|}{CF}}.COOEt$$

$$CH_2F.CO.CHF.COOEt + C_6H_5CH = CH.CO.C_6H_5 \longrightarrow$$



$$CH_2F.CO.CH_2.COOEt + CH_2 = CH.CO.CH_3 \longrightarrow CH_2F.CO.\underset{\displaystyle COOEt}{\underset{|}{CH}}.CH_2.CH_2.CO.CH_3$$

$$CH_2F.CO.CH_2.COOEt + CH_2 = CH.CN \longrightarrow CH_2F.CO.\underset{\displaystyle CH_2.CH_2.CN}{\underset{|}{\overset{\displaystyle CH_2CH_2CN}{\overset{|}{C}}}}.COOEt$$

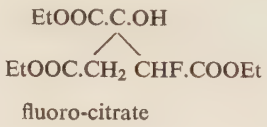
We have studied the relative ability of these compounds to give Michael reactions with the various acceptors, but it would lead too far to enter into a discussion of these data.

The most important of the compounds made available is oxalofluoroacetate.

$$EtOOC.CO.CHF.COOEt + NC.CH_2.COOEt \longrightarrow$$

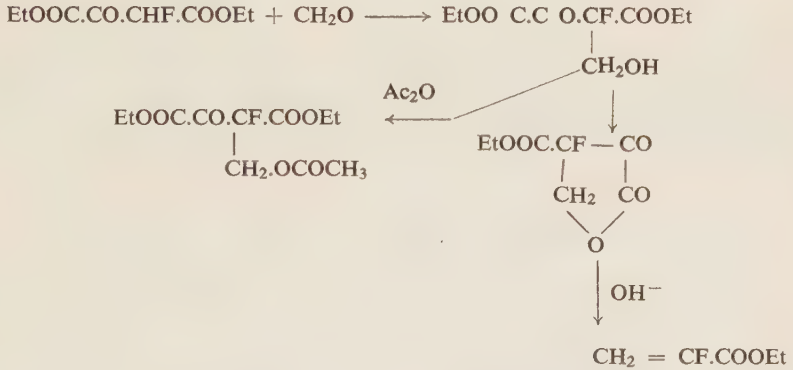
$$\begin{matrix} EtOOC.C.CHF.COOEt \\ \| \\ NC.C.COOEt \end{matrix} \xrightarrow{H^+} \begin{matrix} EtOOC.C.CHF.COOEt \\ \| \\ CH.COOEt \\ \text{fluoro-aconitate} \end{matrix}$$

$$EtOOC.CO.CHF.COOEt + Br.CH_2COOEt \longrightarrow Zn$$

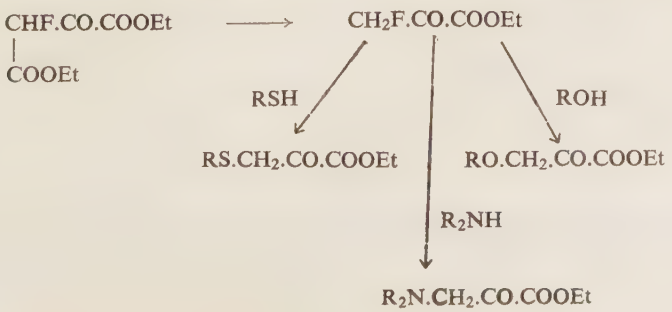


fluoro-citrate

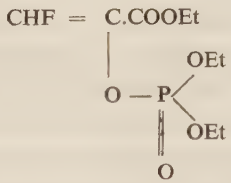
In accordance with its highly ketonic character, it is accessible — as Rivett[45] has shown — to the Reformatzky reaction, so that fluorocitrate is now easily available. With ethyl cyanoacetate, fluoroaconitate is formed in a two-step synthesis. [46] Reduction gives the interesting fluoromalate, or rather the mixture of the two diastereomeric forms. [47, 48] With aldehydes, oxalofluoroacetate reacts in a manner to be expected from the well-studied chemistry of ethyl oxaloacetate.[49, 50]



The primary aldol-like product can be stabilized by acetylation, or it lactonizes to acyclic compound, which under the influence of alkali gives the interesting α-fluoroacrylate — in the case of formaldehyde — or its homologs. Acid treatment transforms oxalofluoroacetate into the important fluoropyruvic acid.[52] Whilst its biological properties will be discussed presently, I would like to stress the curious observation that the fluorine in this acid is unusually sensitive to nucleophilic reagents; it is easily replaced by alkoxy, alkylthio and dialkylamino groups. [53, 54]



Otherwise, the compound behaves normally; for example, it is reduced to fluorolactic acid, from which it can be prepared by oxidation, e.g., with N-bromosuccinimide; [40] it was transformed into its phosphoenol derivative by reaction with triethyl phosphite:[55]



Thus, a certain number of fluoro-analogs of members of the Krebs cycle have become available, most of them having been mentioned already. We hope that we will succeed also in the preparation of the three missing links:

Fluoroacetyl-CoA[56]

Oxalofluoroacetate

Fluoropyruvate

Fluorocitrate

Fluoroaconitate

[Fluorisocitrate]

[Oxalofluorosuccinate]

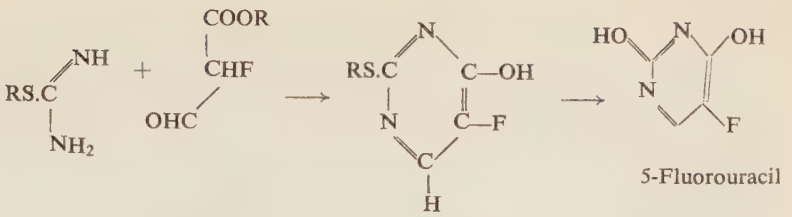
[Ketofluoroglutarate]

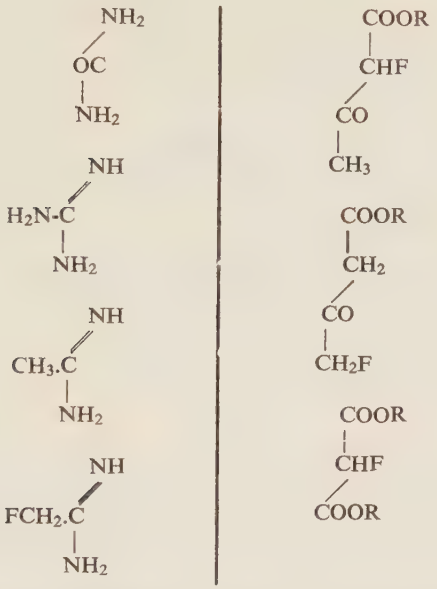
Fluorosuccinate

Fluorofumarate

Fluorolactate

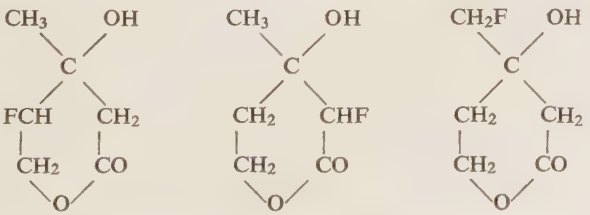
Obviously, the availability of the fluorinated keto-esters opened the way into the pyrimidine series. In the following chart we have schematically summarized our studies.



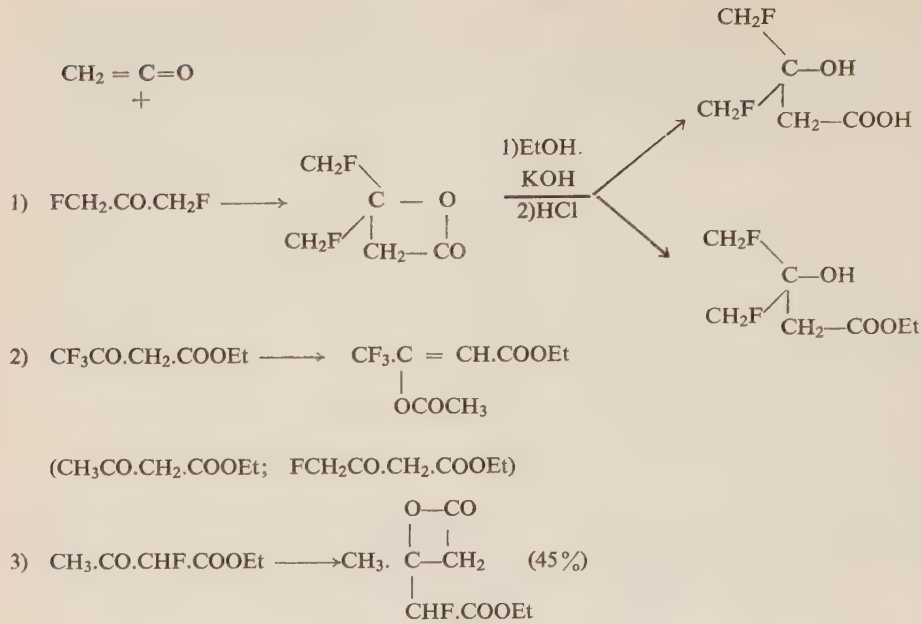


Thus, about 30 fluorinated pyrimidines have been made. Amongst them is one which Heidelberger and his school[57] have described before us. This has proven to be a tumor-inhibiting as well as antibacterial agent of considerable interest; its action is due to the fact that it is incorporated into nucleic acids instead of uracil — as would have been expected.[58–61] The results of tests at present being carried out with other fluorinated pyrimidines will, therefore, be eagerly awaited.

Another cycle, which we have mentioned occasionally, is that of the steroids. We have recently synthesized[32] two of the three possible position-isomeric fluoromevalolactones

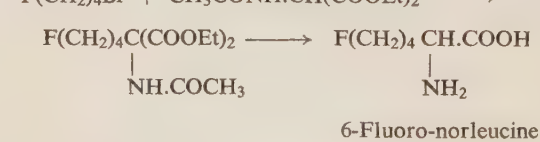


In this connection we have studied the reaction of ketene with some of our fluorinated ketones and keto-esters. Some patents describe a reaction like the first one in the following chart:



However, ethyl acetoacetate as well as the γ-fluoroacetoacetate and the γ,γ,γ-trifluoroacetoacetate gave in our hands only the enol acetates. Only in the case of α-fluoroacetoacetate which is practically not enolized — as we have seen — the desired product has been obtained. [27]

Some first attempts have been made in the amino acid series. In the chart we summarize two reactions described recently by Raasch [63] who synthesized 6-fluoronorleucine and, using fluorobromopropane, 5-fluoronorvaline, and we refer to a curious product which we obtained from phthalimide and ethyl chlorofluoroacetate. [64]



6-Fluoro-norleucine

Its hydrolysis to fluoroglycine, which would contain fluorine and the amino group at the same carbon atom, has not succeeded so far, However, other analogous synthe-

ses are under way, and the biological properties of these amino acids and the corresponding peptides will undoubtedly be of some interest.

This lecture has so far dealt almost exclusively with the chemistry of these new fluoro-aliphatic compounds, and it is appropriate that I mention at least briefly some of the biological properties of the compounds which have been studied by Dr. Avi-Dor and his group at the Israel Institute for Biological Research in Ness-Zionah. Before doing so, I would like to dwell on a very important biological aspect, the question of an antidote to fluoroacetate poisoning. It seemed unlikely that one would exist, in view of the mode of action proposed by Peters which shows that fluoroacetate itself is not toxic, but enters the metabolic pathway and is only transformed *in vivo* into the toxic product, in view also of the low molecular weight and, therefore, easy mobility of the ion. It seemed obvious, however, that massives doses of acetate and potential acetate sources might counteract the effect of fluoroacetate. Indeed, Gitter has found that acetamide protects rats against fluoroacetate poisoning. [65, 66] Why only acetamide is effective is not absolutely clear. It might be due to the fact that acetamide is not hydrolysed; fluoroacetic acid may thus be converted into fluoroacetamide which we have found to be 5-7 times less toxic than fluoroacetic acid itself. [65–68]

Fluoroacetate and fluoropyruvate cause in bacteria (*E. coli*) a growth inhibition, which, however, is only temporary. Fluoropyruvate is 20-50 times more effective than fluoroacetate. [69] On the other hand, fluoropyruvate is 20 times less toxic to mice than fluoroacetate, an observation which — at least superficially — is not in accord with Peter's theory. The poisoning by appropriate doses does not resemble fluoroacetate poisoning. In particular, it does not cause an accumulation of citric acid. Fluorolactate, the reduction product of fluoropyruvate, is a substrate for yeast lactic dehydrogenase and is also non-toxic. [70,72] The non-toxicity of fluoropyruvate, which — as stated — applies only to mammals, may well be due to the lability of its C-F bond. In fact, it has been found that it reacts immediately with SH-compounds, liberating hydrofluoric acid. The compounds formed are characterized by their spectra, which are different if the SH compound has in β-position a free amino group or not. In the former case, e.g. cysteine, the product absorbs at 295-300mμ, in the latter (glutathione, thiomalic, thioglycolic acid) at 265-275 mμ, but this maximum is also shifted to 295-300 mμ by addition of borate. [70,73,74] It is thus possible to determine cysteine and glutathione, e.g., in a mixture. Judging from existing data in the case of *p*-hydroxyphenyl-pyruvic acid, [95] the absorption at 295-300 mμ is due to the enolic form of an α-keto-acid, and this enolization is promoted by borate. If an NH_2 group is present in the β-position to the mercapto group, the product is already largely enolized without borate.

Fluoropyruvic acid, is, however, not transformed quickly enough by this reaction to be without any biological effect at all. It causes a swelling of the mitochondria and produces — indirectly — the biochemical effects connected with this phenomenon.

Here, too, the reaction may be due to an SH-group which is linked normally to the ATP-magnesium complex; this link is destroyed by the acid. [76, 77]

However, the most interesting effect of fluoropyruvate is the differentiation of non-glycolysing and glycolysing cell systems. Both the former, e.g., the chorio-allantoic membrane, and the latter, e.g., tumors and chick fibroblasts, are inhibited completely by 10^{-3}M of fluoropyruvate. The inhibition is overcome in tumours and fibroblasts, but not in non-glycolysing systems, by addition of glucose. One will conclude that the glucose in the glycolysing systems permits a rapid reduction of fluoropyruvate to the non-toxic fluorolactate and thus prevents any irreversible damaging effect of fluoropyruvate. In accordance with this explanation, the glucose effect is abolished by 30×10^{-2}M pyruvic acid, which competes successfully with the fluoropyruvic acid as hydrogen acceptor. In principle, therefore, fluoropyruvate could be used for the selection of cells of desired glycolytic activity. [78]

The detailed biochemical study of the new fluorine compounds is only in its beginning. Interesting observations have been made as regards the toxicity of these substances, but their enumeration here would not appear to be of particular interest. It remains only to thank my collaborators, especially Sasson Cohen and Israel Shahak, for their help; they have laid the foundation for a new field of chemistry which, I am convinced, will yield both theoretically and practically interesting results.

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THE THERMAL HYDROLYSIS OF METAL CHLORIDES. III. MAGNESIUM CHLORIDE

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ABSTRACT

Some information on the mechanism of hydrolysis of magnesium chloride has been obtained. Starting with a hydrated sample, the main hydrolytic reaction is a first order decomposition of magnesium chloride monohydrate, whereby magnesium hydroxy-chloride is formed. Owing to a simultaneous dehydration of the salt, the residue obtained at the end of a run contained about 10%, or less, magnesium chloride.

The hydrolysis of anhydrous magnesium chloride is initially a zero order surface reaction with a low activation energy (2.9 K cal/mole). This reaction is complicated by: a) the formation of magnesium chloride monohydrate, when the flow gas contains water vapour at a higher pressure than the equilibrium pressure of this salt, and b) the formation of an inert magnesium hydroxy-chloride crust on the surface of the reacting solid.

At temperatures above 400°C the magnesium hydroxy-chloride first formed may be further hydrolysed to magnesium oxide, but only after a slow transformation into its components. This reaction requires a high activation energy of 45.6 K cal/mole.

INTRODUCTION

The partial or complete hydrolysis of magnesium chloride has attracted great interest, and formed the subject matter of many publications since the beginning of the century[1] until recent years[2,3,4]. Some authors were most interested in the preparation of the anhydrous salt and the prevention of the hydrolytic reaction, others put the emphasis on the equilibrium states attained at various temperatures and the thermodynamic values involved. The available literature up to 1944 has been critically reviewed by Kelley[5].

When the hydrated salt is heated, partial hydrolysis occurs as the two last molecules of water of crystallization are driven off, at approximately 160°–180°C. Some magnesium hydroxy-chloride [Mg(OH)Cl] is formed, which appears to be stable[2] up to 500°C. The exact composition of the solid products obtained at various temperatures was not determined, and records in the literature vary widely. Moreover, no information seems to be available either on the rate of hydrolysis or its mechanism.

Previous work by the present authors on the hydrolysis of other metal chlorides[6,7] gave grounds to believe that by using the same methods of investigation, the kinetics and mechanism of the hydrolysis of magnesium chloride could also be elucidated. Actually, results were sometimes erratic, and in some cases it is difficult to derive

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quantitative conclusions from them. Yet, considering the wide technical interest of the subject, it is thought that much of the information obtained is of practical value.

EXPERIMENTAL

The magnesium chloride hexahydrate used was a "Baker Analyzed" reagent. The anhydrous salt was prepared by dehydrating this product with thionyl-chloride[8]. Details of the apparatus and the experimental technique have been given in previous articles [6,7].

RESULTS

1. *Magnesium chloride hexahydrate* ($MgCl_2.6H_2O$)

Runs were made in the temperature range 180°–280°C, in a porcelain boat, with most samples weighing about 100 mg, a flow rate of 34 ml/min. of nitrogen and water vapour pressure of 26 mm Hg. The form of the curves obtained is illustrated in Figure 1. After an induction period of 10 to 20 min. during which most of the water of

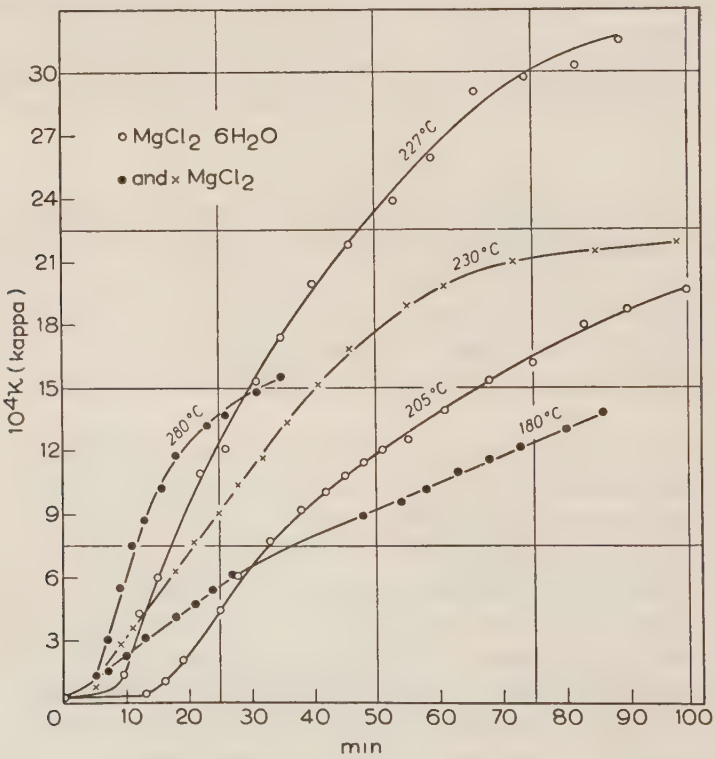

Figure 1
Typical hydrolytic curves of hydrous and anhydrous magnesium chloride in the temperature range 180°-280°

crystallization evaporated, there was a steep but curved rise in conductivity, indicating a reaction of the first order. When about 40% of the total chloride content of the samples had evolved, the curves flattened off, so that the residue was not completely converted to the hydroxy-chloride, although water vapour in the flow gas was driven over it. In Figure 2 are given a few log $(a\text{-}x)$ versus time plots (a =

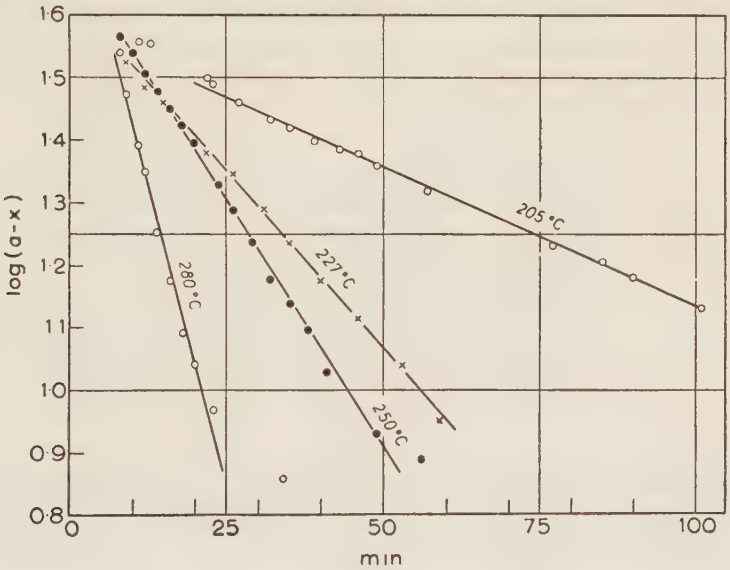

Figure 2.
Some first order hydrolytic curves of magnesium chloride hexahydrate, log $(a\text{-}x)$ vs. t (a is the expected conductivity when sample is hydrolysed to magnesium hydroxy-chloride).

expected conductivity for a complete conversion to magnesium hydroxychloride, and x = measured conductivity at time t min), showing that the first order equation holds up to 70% of the reaction. From the slopes of these plots the reaction constants k_1 were calculated. Heaping the sample in the boat, doubling the weight of the sample, or increasing the rate of flow of the gas, produced only minor changes in the constants k_1, within the limits of the observed reproducibility of experiments under identical conditions. This is in accord with a first order reaction in which the last molecule of water of crystallization is mainly responsible for the hydrolytic reaction.

The logarithm of the constants k_1 versus the inverse of absolute temperature lie on a straight line (Figure 3), from the slope of which the energy of activation 16.6 Kcal/mole was calculated. This is very nearly equal to the heat of reaction necessary for the conversion of the monohydrate into the hydroxy-chloride[5]:

$$MgCl_2.H_2O \longrightarrow MgOHCl + HCl + \triangle H \qquad (1)$$

$$230.3 = 191.3 + 22.0 + 17 \text{ Kcal.}$$

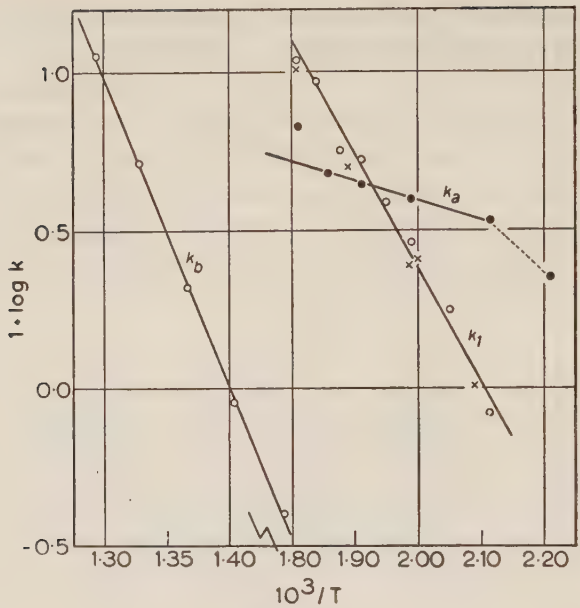

Figure 3

Temperature gradients of rate constants $\log k$ vs. $10^3/T$. k_1, for magnesium chloride hexahydrate $(2 + \log k)$; (k_a, for hydrolysis of the anhydrous salt to magnesium hydroxy-chloride, x and o — are points of two series of experiments carried out at widely different times); k_b, for complete to hydrolysis of magnesium oxide.

Hence the hydrolytic reaction may be identified with equation. (I)

Simultaneously, some of the monohydrate is dehydrated:

$$MgCl_2.H_2O \longrightarrow MgCl_2 + H_2O \qquad (2)$$

an endothermic reaction requiring about 19.3 Kcal/mole. The final product is therefore a solid solution, or mixture, of magnesium chloride in magnesium hydroxy-chloride.

2. *Anhydrous Magnesium Chloride*

Runs with the dehydrated salt were carried out in two ranges of temperatures: between 180°–280°C and 400°–500°C. The hydrolysis curves exhibited a short delay of 2–5 min. (probably due to the heating up of the sample to the controlled temperature), followed (see Figure 1) by a linear increase in conductivity up to 20–35% of the total amount of chloride in the sample (depending on the temperature). This part of the hydrolysis curve is similar to those obtained with cupric chloride and other anhydrous metal chlorides, and may be represented by a zero order constant k. The straight line bends at an early stage into a flattened curve; with experi-

ments below 300°C the evolution of hydrogen chloride stops at this stage, but with experiments above 400°C another zero order reaction follows after a clearly defined induction period. The latter is shortened as temperature rises, and at 500°C the first and second linear parts of the curve coalesce into a single straight line (see Figure 4).

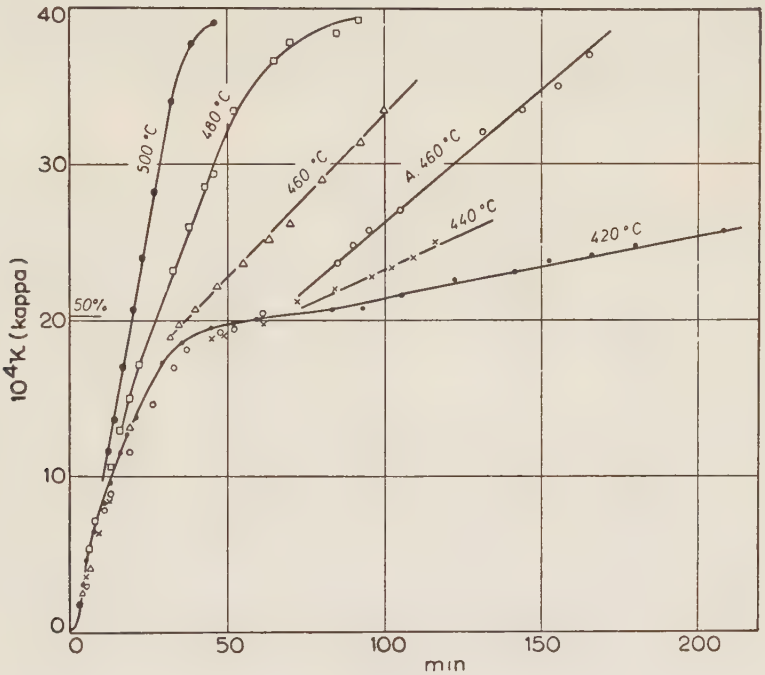

Figure 4
Hydrolytic curves of anydrous magnesium chloride in the temperature range 400°–500°C. Wt. of samples 30 mg, flow rate 68 ml/min. With a flow rate 34 ml/min. 50% indicates conversion of a 30 mgr. sample into Mg(OH)Cl.

(a) *Low temperature runs*, 180°–280°C

Most of the experiments were carried out in platinum boats (length 15 mm), with a flow of gas 34 ml/min., containing water vapour at 26 mm Hg pressure, and samples weighing 34 mg. The conductivity versus time curves were rather complicated. At 180° there is a break on the linear curves, and the reaction is slowed down (see Figure 1). The curves obtained are the most regular at 230°–250°C, about 85% of the samples being converted into the hydroxy-chloride. At higher temperatures the curves deviate from the linear course at a rather low percentage of hydrolysis, suggesting the formation of an inert crust of magnesium hydroxy-chloride.

In Table I are recorded the weights of the magnesium chloride and the magnesium hydroxy-chloride in the residue, calculated from the percentage of hydrolysis, as

measured by conductivity. The comparison of the total to the actual weight of the residues (weighed in most cases towards the end of the hydrolytic reaction) is quite satisfactory: in experiments with the hexahydrate, the actual weight tends to be somewhat higher, whilst in experiments with the anydrous salt, somewhat lower, than the calculated weight. This is probably due to the absorption of 2–3% moisture by the anhydrous samples. Table I thus proves that the conductivity measurements give a correct value of the extent of hydrolysis, and the complicated nature of the hydrolytic curves is caused by a multiplicity of factors whose relative influence varies with increasing temperature.

TABLE I
Weights of hydrolysed residues

Wt. of sample mg	Temp. °C	% of hydro-lysis	Wt. of residue	Composition and total wt., calc.		
				MgOHCl	$MgCl_2$	Total
*108.2	250	82.0	43.9	33.52	9.12	42.64
*107.6	255	81.7	42.7	33.26	9.23	42.49
*101.0	280	79.5	40.0	30.34	9.69	40.03
32.5	200	86.2	28.4	22.59	4.49	27.08
28.9	230	78.8	22.3	18.37	6.13	24.50
35.3	230	83.9	28.4	23.86	5.68	29.54
34.0	230	87.0	27.5	23.84	4.42	28.26
32.5	250	79.3	27.0	20.79	6.73	27.52

* The first three samples were the hydrate $MgCl_2.6H_2O$, all the rest the anhydrous salt.

The water vapour pressures of the monohydrate recorded by Kelley[5] are: 3.3 and 29.2 mm Hg at 178°C and 227°C respectively. Evidently, when the vapour pressure in the flow gas is higher than that of the monohydrate, the latter compound is formed, and the hydrolysis proceeds at a slower rate according to reaction (1), causing the break in the curves at 180°C. At increasing temperatures, when the vapour pressure of the monohydrate rises above 26 mm, the slightly exothermic reaction (3) takes place:

$$MgCl_2 + H_2O \longrightarrow MgOHCl + HCl + \triangle H \qquad (3)$$
$$153.2 + 57.8 = 191.3 + 22.0 - 2.3 \text{ Kcal.}$$

but, as already mentioned, the magnesium hydroxy-chloride formed intervenes with the smooth reaction by reducing the exposed surface of the reactant. This disturbing effect is the more pronounced the higher the temperature. Reduction of the water vapour pressure in the flow gas produces a similar effect.

The exposed surface area of the samples, the weight of the samples, and the flow rate, each affect the linear portion of the hydrolytic rate (expressed by $10k$) to about the same extent as in Table II. The weight effect, which was absent in the case of the anhydrous salts previously studied[6,7], indicates that water vapour is also adsorbed to some extent by the "body" of the samples and not only by their surface.

TABLE II

Variation of $10k$ *with spread and weight of samples, and flow rate*

230°	Wt. of sample	35.3	34.0[a]	77.7	33.1[b]
	$10k$	3.93	5.21	5.04	5.31
250°	Wt. of sample	32.5	30.5[a]	77.8	33.4[b]
	$10k$	4.45	6.50	5.79	5.36

a = porcelain boat; b = 68 ml/min.
All other runs in platinum boat and 34 ml/min. flow rate.

From an Arrhenius plot of $10k$ (see Figure 3), in the temperature range 200–265° (where distinct linear hydrolytic curves were obtained) the activation energy 2.9 Kcal/mole was calculated. As already noted, reaction (3) is slightly exothermic[4,5], and hence the very low activation energy observed should be accounted for as caused by the diffusion of hydrogen chloride into the flow gas.

(b) *High temperature runs*, 400–500°C

Two series of runs were made in this range of temperatures with flow rates of nitrogen of 34 and 68 ml/min. respectively. Platinum boats were used, the water vapour pressure was 26 mm Hg, and the samples weighed about 30 mg. The hydrolytic curves of one of these series, with the higher flow rate, are shown in Figure 4. A single curve from the other series (presenting the same general aspect) is also given for comparison.They clearly indicate two consecutive reactions with the dividing line at about 50% hydrolysis. The lower parts of the curves resemble those obtained with anhydrous magnesium chloride in the temperature range 230°-250°C, and represent the same reaction, (3), in which magnesium hydroxy-chloride is formed. The initial rate of this step changes only slightly with temperature, $10k_a$ being between 6.0 to 7.5, as compared to 5.36 at 250°C (see Table II). This is in harmony with the very low activation energy of this reaction.

The second zero order reaction takes place only after a well defined induction period. The weights of the residues indicate that the final product is *magnesium oxide*. Hence it is suggested that the cause of the induction period is a slow decomposition of the magnesium hydroxy-chloride, formed in the first step, into magnesium chloride and magnesium hydroxide, the latter being instantly converted into the oxide:

$$2Mg(OH)Cl \longrightarrow MgCl_2 + Mg(OH)_2 \longrightarrow MgCl_2 + MgO + H_2O \qquad (4)$$

The magnesium chloride thus freed from the very stable hydroxy-chloride undergoes a further cycle of hydrolysis at a rate given by the linear slope k_b. It is of interest to note that at 500°C, the instability threshold of magnesium hydroxy-chloride, a single undivided curve is obtained.

From the Arrhenium plot of k_b (see Figure 3), the very high energy of activation

45.6 Kcal/mole is calculated; this is the energy required for the rearrangement of the stable magnesium hydroxy-chloride according to equation (4).

CONCLUSIONS

1. On heating magnesium chloride hexahydrate in a gas current, five molecules of water are driven off before any appreciable amount of hydrolysis occurs.
2. The monohydrate undergoes two simultaneous reactions:

$$\text{a)}\quad MgCl_2.H_2O \longrightarrow Mg(OH)Cl + HCl$$

$$\text{b)}\quad MgCl_2.H_2O \longrightarrow MgCl_2 + H_2O,$$

   the first order hydrolytic reaction a) requires a lower energy of activation and is therefore the faster. The residue is a solid solution of magnesium chloride in magnesium hydroxy-chloride.
3. Anhydrous magnesium chloride may be hydrolysed in a current of gases containing water vapour according to a zero order reaction. Magnesium hydroxy-chloride is formed first which is very stable and inert up to 500°C.
4. If the water vapour pressure in the flow gas is higher than the equilibrium pressure of magnesium chloride monohydrate, the absolute rate of the hydrolytic reaction is slowed down by the formation of the monohydrate, as evidenced, for example, by experiments at 180°C (see Figure 3).
5. A crust of the inert magnesium hydroxy-chloride formed around the anhydrous magnesium chloride grains may reduce the initial rate of hydrolysis quite appreciably.
6. At temperatures above 400°C a very slow transformation of the magnesium hydroxy-chloride into its components, the chloride and the hydroxide may take place. This occurs at a temperature above that of the decomposition of the hydroxide into the oxide (350°C), and requires a high energy of activation, 45.6 Kcal/mole.
7. Above 400°C the hydrolysis may therefore proceed rather slowly until the final stage of magnesium oxide. This, also, is a zero order reaction.
8. At 500°C the transformation of the magnesium hydroxy-chloride becomes fast enough to give a single zero order hydrolytic reaction of magnesium chloride into magnesium oxide.

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PREPARATION OF PENTAMETHYLENE DIBROMIDE AND CHLOROBROMIDE

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In the course of a synthetic study relatively large quantities of pentamethylene dibromide and chlorobromide were required.

The dibromide can be prepared by the action of fuming hydrobromic acid on pentamethylene glycol[1] or 1,5–diphenoxypentane[2], and by the action of hydrobromic and acetic acid on 1.5–diisoamyloxypentane[3], and more conveniently from benzoylpiperidine and phosphorus pentabromide (yield, 62–75%) [4] or from tetrahydropyran and hydrobromic acid (yield, 80%)[5]. We have found that the reaction of tetrahydropyran with sodium bromide and sulphuric acid is equally possible and perhaps even easier.

As to the preparation of tetrahydropyran, the catalytic hydrogenation of the easily available dihydropyran is the method of choice. The use of Raney nickel as catalyst has been described[6], and is rather cumbersome. Our experience shows that 10% palladium on charcoal is effective and requires no special precautions.

Also the previous syntheses of pentamethylene chlorobromide have been based on tetrahydropyran and consisted of two steps: the conversion into pentamethylene chlorohydrin[7] or its acetoxy derivative[8,9], and their treatment with phosphorus tribromide or hydrobromic acid. We have found that the treatment of tetrahydropyran with a mixture of sodium chloride, sodium bromide and sulfuric acid gives in a single step a 35% yield of the desired chlorobromide which is accompanied by a 15% yield of pentamethylene dibromide.

EXPERIMENTAL

Tetrahydropyran. In a low pressure hydrogenation apparatus 42 g of dihydropyran and 0.4 g of 10% palladium on charcoal were hydrogenated with 0.5 mole of hydrogen (about 20 minutes). The mixture was filtered and could be used for the following syntheses without distillation.

Pentamethylene dibromide. To a stirred mixture of 43 g (0.5 mole) of tetrahydropyran, 125 g of sodium bromide and 150 ml of water, 190 ml of concentrated sulphuric acid was added at such a rate the temperature did not exceed 75°. The mixture was heated with vigorous stirring at 120° for 3 hours. The solution was then poured into 2 liters of an ice - water mixture and separated and the acidic layer extracted with ether. The organic layer was washed with sodium carbonate solution, and water dried over magnesium sulfate. The ether was evaporated, and the residue

combined with the main fraction and distilled under reduced pressure to yield 106 g (92%) of pentamethylene dibromide; b.p. 107/24 mmm., n_D^{25} 1.5103, in agreement with reported values[10].

Pentamethylene chlorobromide. To a stirred mixture of 62 g of sodium bromide, 38 g of sodium chloride, 43 g of tetrahydropyran and 200 ml of water, 190 ml of concentrated sulfuric acid was added dropwise at 75°. The mixture was stirred and refluxed for 10 hours at 110°, poured into 2 liters of an ice–water mixture and extracted with ether. The ether solution was washed with sodium carbonate and sodium bisulfate solution dried over magnesium sulfate and concentrated. The product was distilled with an efficient fractionating column under reduced pressure, and gave 32 g (35%) of pentamethylene chlorobromide b.p. 102–104/30mm; n_D^{25} 1.4815[8,9] and 17 g (15%) of pentamethylene dibromide.

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Triglyceride release by liver slices

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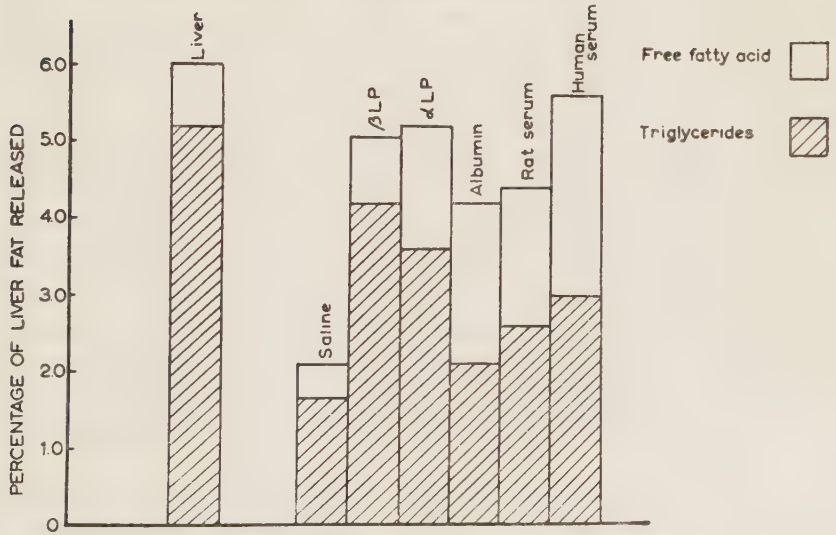
Work done in this laboratory has shown that the liver assimilates plasma triglycerides on one hand and on the other releases triglycerides into the blood[1]. Liver and blood plasma may therefore be looked upon as a common pool of triglyceride which equilibrates rapidly. This pool is constantly fed by the free fatty acids which are released from adipose tissue and are converted into liver triglycerides. Material is removed from the pool by combustion, conversion to phospholipids and also by return to adipose tissue storage.

Perfusion experiments have shown that considerable amounts of fatty acids are

TABLE I

LIPID RELEASE INTO DIFFERENT MEDIA



released from the liver, contrary to the free fatty acid released from adipose tissue, those released by the liver were predominantly in the form of neutral glycerides. It has now been found that this release could also be studied *in vitro* with slices prepared from livers which have been charged with labelled palmitic acid. In these

experiments the predominance of triglycerides as the form of release from the liver was again confirmed when α and β lipoprotein solutions were used as the incubation medium. Incubation in serum or albumin however, brought about a considerable release of free fatty acids. While a limited release was obtained when saline served as incubation medium, plasma lipoproteins increased this release markedly (Table 1). The system was found to come to rapid equilibrium at which no further release is obtained, unless the medium is changed. When unlabelled liver slices were introduced into the radioactive medium, large portions of the labelled fatty acids were taken up.

Incubation of liver slices in serum or lipoprotein media containing labelled triglyceride, resulted in the uptake of the triglycerides by the liver.

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Studies on gangliosides of Tay-Sachs brain

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Tay-Sachs disease (infantile amaurotic familial idiocy) is a fatal, hereditary primary lipid storage disease. It is characterized by an abnormal accumulation in brain of gangliosides, which are water-soluble, high molecular weight glycolipids containing sphingosine, fatty acid, hexose, hexosamine and sialic acid. While studying the chemical basis for this derangement of glycolipid metabolism, differences were observed in the solubility properties of Tay-Sachs and normal brain gangliosides.

When chloroform-methanol extracts of normal brain were washed with aqueous-methanolic solutions containing KCl[1], all gangliosides were partitioned into the aqueous upper phase. However, when chloroform-methanol extracts of Tay-Sachs brain were similarly treated, only 80% of the gangliosides (ganglioside "A") were thus taken into the water phase.The remainder (ganglioside"B") could be extracted into the upper phase only by further washings with media containing water instead of KCl. When these two ganglioside fractions were purified on columns of silicic acid, the chemical composition of the purified preparations was found to be almost identical.

While investigating the unique solubility behavior of Tay-Sachs gangliosides, it was observed that both normal and Tay-Sachs ganglioside "A" in aqueous solution, could be made chloroform soluble in the presence of KCl and various lipid fractions. Under similar conditions ganglioside "B" became chroloform-soluble even in the absence of added lipids. It may be assumed that in chloroform-methanol extracts of brain, gangliosides are complexed with either lipids or proteins. These complexes are chloroform soluble and their formation is facilitated by the addition ofsalt.

The nature of the complexing agents and the reason for the presence of ganglioside "B" in extracts of Tay-Sachs and not of normal brain are currently under investigation.

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Identification of a new class of brain glycolipids

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The glycolipids hitherto identified in brain tissue are, a) cerebrosides, which contain equimolar quantities of sphingosine, fatty acid and hexoses, and b) gangliosides, high molecular weight lipids containg sphingosine, fatty acid, hexose, hexosamine and sialic acid. A new class of brain glycolipids, intermediate in composition between these two, has now been isolated in the following manner. When chroloform-methanol lipid extracts of Tays-Sachs brain were washed with aqueous-methanolic mixtures[1] to remove all water-soluble gangliosides, the washed extracts still gave a positive reaction for hexosamine. In order to isolate and identify the hexosamine-containing lipid, the washed extract was chromatographed on a column of silicic acid and the lipids eluted by applying a gradient of chloroform with increasing concentrations of methanol. Eluent fractions were assayed for both hexose and hexosamine. Two hitherto unknown brain glycolipids were isolated by this procedure. The first was identified as a sphingosine-fatty acid-tri-hexoside containing one mole of hexosamine per mole of sphingosine[2]; the second as a sphingosine-fatty acid-dihexoside without hexosamine. Neither of these compounds could be detected in normal humain brain. However, a third glycolipid containing sphingosine, fatty acid and hexose was isolated in small quantities from normal human brain. A complete chemical analysis of this glycolipid has not yet been performed, but when chromatographed on silicic acid-impregnated paper, it migrated with an Rf different from either cerebrosides or the two glycolipids of Tay-Sachs brain.

Isototope tracer studies have indicated that the two new glycolipids from Tay-Sachs brain have a turnover similar to that of gangliosides and cerebrosides, but considerably lower than that of lecithins and cephalins.

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Mechanism of epinephrine effect on fatty acid esterification and release in adipose tissue

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The *in vitro* incorporation of 1–^{14}C–labeled fatty acids (FA) into the triglyceride esters (FE) of rat epididymal fat tissue is considerably decreased in the presence of epinephrine, when determined after an incubation of 1 to 2 hours. At the same time epinephrine causes the release of non-labeled FA due to lipolysis of tissue FE, thus lowering the specific activty of medium FA from which incorporation is measured. When sections of fat tissue were incubated for shorter time intervals (Table I) the incorporation of the label from control media proceeds almost linearly with time. In the presence of epinephrine, however, there is at first a brief period of enhanced uptake, followed by a stage of markedly retarded incorporation. Lipolysis, as evident from rising quantities of FA and glycerol in the medium predominates only in the latter stage.

TABLE I

	Minutes of incubation	Incorporation in tissue FE counts/min./g.x10^3	Medium* FA release μEq./g.	FA specific activity counts/μEq./g. x10^3	Glycerol release μMoles/g.	FA/Glycerol ratio increment
Control	10	9.2	0.00	202	0.04	0.00
Epinephrine		14.3	0.00	200	0.09	0.00
Control	20	17.8	–0.10	221	0.10	0.00
Epinephrine		15.8	0.90	102	0.76	1.38
Control	60	38.0	–0.05	205	0.24	0.36
Epinephrine		20.5	2.95	48.3	1.62	2.38
Control	120	65.8	0.15	160	0.35	1.82
Epinephrine		22.5	4.75	32.9	2.26	2.82

* 250 mg sections of tissue were incubated each in 2 ml of 5% albumin solution, *p*H 7.4, containing 0.95 μEq. 1-^{14}C-palmitate of specific activity 204,000 counts/μEq. with or without 2 μg epinephrine hydrochloride.

Epinephrine *in vitro* promptly activates phosphorylase in adipose tissue[1] with resultant glycogenolysis and lactic acid production. It appears then, that the stimlation of esterification during the short initial interval of incubation is brought about by augmenting the flow of glucose metabolites which as precursors of the glycerol moiety promote the incorporation of the FA into the tissue glycerides.

The reduced incorporation of the label occurring later, cannot be attributed to the dilution of medium 1–^{14}C–FA with newly released non-labeled FA, since increase in medium FA content would be expected to elicit a coresponding rise in esterifi-

cation[2]. That the esterification reaction is actually inhibited may be further inferred from the rising ratio of FA to glycerol flowing out from the tissue, showing loss in ability to re-esterify FA.

Mobilization of tissue fat through inhibition of esterification and increase in lipolysis seeems therefore to take place only after the initial, epinephrine induced depletion of its glucogenic stores.

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C-methylpurines as substrates of mammalian xanthine oxidase (XO)

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1. The antitumor agent 6-methylpurine (I) is characterised by its high toxicity which has been ascribed to the lack of a biochemical mechanism for its detoxification[2]. We have however found that I is attacked by XO according to the following scheme:

6-Methylpurine ——→ 6-methyl-8-hydroxypurine —→

6-methyl-2,8-dihydroxypurine

2. 8-Methylpurine behaves like purine, i.e., it is converted first into 8-methyl -hypoxanthine and subsequently into 8-methylxanthine. The first step proceeds at about 1/6 of the rate of oxidation of purine, while the rate of the second step is equal to conversion of hypoxanthine to xanthine.

8-Phenylhypoxanthine is not attacked by the enzyme.

3. 2-Methylpurine likewise is converted first to 2-methylhypoxanthine. However, in contrast to hypoxanthine, which is not attacked at all at C-8[3], its 2-methyl derivative is oxidised at this position, albeit at a very small rate.

These observations lead to new conclusions concerning the mechanism of action of XO.

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The effect of pyrimidine analogues on the regulatory mechanisms in the cell

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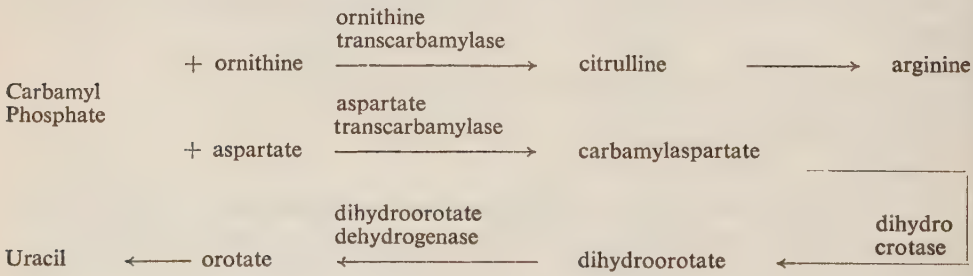
In a study of the mode of action of 2–Thiouracil (2–TU), it has been observed that cells of *E. coli* grown in the presence of arginine (or citrulline) and 2–TU, lose their ability to form colonies on solid medium. However, if 2–TU is added to the solid medium, all the cells are able to form colonies.

These phenomena were found to be correlated with changes in the level of the enzymes acting in the biosynthesis of arginine and uracil. These syntheses are metabolically related and are controlled by negative feedback mechanisms.

It has been shown that the conditions under which loss of viability occurred were: repression of the enzyme ornithine transcarbamylase by arginine (or citrulline) and a considerable rise in the level of the enzyme aspartate transcarbamylase caused by the analogue.

6-azauracil and 5-fluorouracil were also tested and it was found that the decrease, in colony forming cells occurred only in the presence of 6-azauracil which causes an increase in the level of asparate transcarbamylase.

The significance of these results will be discussed.



Pyrimidine biosynthesis in the presence of carcinogenic concentrations of ethyl carbamate (urethan)

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Work in several laboratories has revealed an inhibitory effect of thymine and/or thymidine on the chromosome breaking[1], carcinogenic[2] and carcinoclastic[3] properties of urethan. Thymine has also been shown to reduce the number of spontaneous lung adenomas in strain A mice[4]. Our study of the incorporation of thymine–2–C^{14} in mouse liver showed that the small but significant incorporation in DNA thymine[5] is doubled in the presence of urethan. This result led to studies of orotic acid–6–C^{14} incorporation.

The constancy of the ratio of incorporation of orotic acid–6–C^{14} in uracil and cytosine of mouse liver RNA, whether urethan is present or absent, does not support the suggested effect of urethan on the transamination reaction which produces a cytosine moeity from a uracil moeity[3]. An increase of 20 per cent in the incorporation of orotic acid–6–C^{14} in liver DNA thymine, in the presence of urethan, seems to eliminate the possibility of a general inhibition by urethan of the transmethylation of a uracil moeity[2].

The possibility of a competitive interaction between urethan and carbamyl phosphate[3] was tested. We found no inhibition of urethan catabolism by mouse liver brei[5] in the presence of a 9 times molar excess of carbamyl phosphate. The *in vitro* effect of urethan on transcarbamylation enzymes and enzymes involved in o otic acid synthesis was tested. Systems contained a final urethan concentration of 0.06M (urethan: substrate ratio$>$30:1) in at least two enzyme concentrations at which activity was proportional to crude rat enzyme preparation added. No effect was found on liver ornithine transcabamylase or carbamyl phosphate synthetase[6], aspartate transcarbamylase[6] or dihydroorotase from deciduoma, or liver dihydroorotate dehydrogenase. This does not support the suggestion of an inhibition by urethan at one of the steps leading to orotic acid formation[2].

The orotic acid pathway of pyrimidine biosynthesis has thus been investigated by a combination of *in vivo* tracer techniques and *in vitro* enzyme inhibition studies without revealing a specific site of urethan action. This suggests that the increase in thymine and orotic acid uptake in the presence of urethan may be a non-specific (anesthetic) effect unrelated to the carcinogenic action of urethan. A difference was found in the pattern of incorporation of these precursors between spleen and liver, lung, skin or brain. This correlates with urethan's leukopenic action, and offers the working hypothesis that organ specific differences in nucleic acid metabolism may be responsible for some of the biological specificities of urethan.

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The enzymatic activities of pepsin obtained from iodo- or azo-pepsinogen

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Iodination of the tyrosine phenol groups of pepsin has been shown by Herriott[1]

to reduce the proteolytic activity of the enzyme. In pepsinogen the tyrosine groups essential for catalytic activity may be protected and, therefore, iodination or coupling reaction will permit substitution of only those tyrosine residues which are unessential for enzymatic activity. Iodo- and azo-pepsinogen were prepared with the object of studying how many iodine or azo groups can be introduced into pepsinogen molecules without changing their potential peptic activity.

Iodo-pepsinogen — The iodination of pepsinogen[2] was carried out in sodium phosphate buffer 0.2 M, *p*H 7.5, with a calculated amount of I_2–KI solution at 4 °C. In some of the experiments radioactive iodine was used. The iodinated pepsinogen was precipitated with $(NH_4)_2SC_4$, redissolved in 0.05 M sodium phosphate buffer, *p*H 7.5, and dialyzed overnight against the same buffer. The iodine content of pepsinogen was measured either by determining the radioactivity of the labelled pepsinogen or by chemical analysis. Diiodotyrosine was determined quantitatively after total hydrolysis according to Mandl and Block[3]. It was found that all the iodine content of iodopepsinogen could be accounted for as diiodotyrosine. The iodinated pepsinogen was activated and pepsin formed assayed by Anson's method[4], and then compared with that of a pepsinogen control similarly activated. It was found that activation of the iodinated pepsinogen containing about 6 atoms of iodine per molecule gives a product with an enzymatic activity equal to that of the control.

Azo-pepsinogen — Pepsinogen was coupled with diazosulphanilic acid in 0.2 M sodium phosphate buffer *p*H 7.5 at 4°. The azo-pepsinogen was precipitated with $(NH_4)_2SO_4$, dialyzed and lyophilized. The number of azo groups introduced to pepsinogen was measured spectrophotometrically according to Tabachnick and Sobotka[5], and by determining the sulphur content of the protein. The potential activity of the azo-pepsinogen was determined and found to be identical with that of pepsinogen control, if the number of azo groups introduced to the molecule is about 6 per protein molecule.

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A new class of trypsin substrates

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Trypsin is capable of cleaving derivatives of the natural amino acids L-lysine and L-arginine[1], as well as esters of ε-amino caproic acid[2].

Recently, it was shown that a polymer of S–(2 aminoethyl) cysteine, which is an analog of lysine, is split by the enzyme in a similar manner to polylysine[3].

In the present study other analogs of lysine and ε-amino caproic acid were prepared

in which polar groups such as ester and peptide bonds replaced methylene groups in the side chain.

Thus β-alanyl glycine ethyl ester (I) was cleaved at the same rate as ε-amino caproic ester (II)

(I) $H_3\overset{+}{N}CH_2CH_2CONHCH_2COOEt$

(II) $H_3\overset{+}{N}CH_2CH_2CH_2CH_2CH_2COOEt$

Furthermore, N,O-diglycyl-DL-serine ethyl ester (III) and N-benzoyl-O-glycyl DL-serine ethyl ester (VI) were hydrolyzed at a rate comparable to that at which lysine esters (V) were split.

(III) $H_3\overset{+}{N}CH_2CO.OCH_2CH(NHCOCH_2\overset{+}{N}H_3)COOEt$

(IV) $H_3\overset{+}{N}CH_2CO.OCH_2CH(NHCOC_6H_5)COOEt$

(V) $H_3\overset{+}{N}CH_2CH_2CH_2CH_2CH(\overset{+}{N}H_3)COOEt$

This shows that extensive modification of the lysine side chain does not affect significantly the ability of the analogs to serve as trypsin substrates. It appears that a compound should possess an ammonium group located at the sixth atom from the susceptible bond, in order to serve as a trypsin substrate; the nature of the intervening groups, however, seems to be of secondary importance.

The findings recorded may be of use in the cleavage of proteins at the carbonyls of the serine or cysteine residues.

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Preparation of some water-insoluble proteolytic enzyme derivatives

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The method of Bar-Eli and Katchalski for the preparation of water-insoluble trypsin[1] was extended to the preparation of water-insoluble derivatives of chymotrypsin, papain and pepsin.

Water-insoluble chymotrypsin was prepared as follows: Chymotrypsin was used as an initiator for the polymerization of DL-tyrosine N-carboxyanhydride to give polytyrosylchymotrypsin (PTC). The latter contained 7% more tyrosine by weight than intact chymotrypsin and retained 60% of the original enzymatic activity. Water-insoluble chymotrypsin (IPTC) was obtained by coupling PTC with the diazonium salt of a copolymer of *p*-aminophenylalanine and leucine[1]. The IPTC prepared, contained 22% protein by weight and possessed 11% of the enzymatic activity

of chymotrypsin and 18% of the hydrolytic activity of PTC. The activity was determined by using tyrosine ethyl ester as substrate. When intact chymotrypsin was coupled directly to the diazotized copolymer, only 4% of the original activity of the enzyme was retained in the final water-insoluble product obtained.

Since papain contains 13% tyrosine by weight[2] and two thirds of the molecules are not essential for enzymatic activity[3], the enzyme was coupled directly to the diazonium salt of the *p*-aminophenylalanine-leucine copolymer. The insoluble product contained 28% protein by weight, and revealed enzymatic activity equivalent to 70% of that of soluble papain, using benzoylarginine ethyl ester, gelatin and human serum albumin as substrates for comparison.

Since pepsin is unstable at *p*H 7.5 at which the coupling reaction should be carried out, insoluble pepsin was prepared by direct coupling of pepsinogen (which is stable up to *p*H 9) with the diazonium salt of the *p*-aminophenylalanine-leucine copolymer, followed by the usual acid activation[4] of the water-insoluble proenzyme to water-insoluble pepsin. The water-insoluble pepsin contained 10% protein by weight. The latter had about 70% of the enzymatic activity of soluble pepsin as measured by the haemoglobin test[5].

Acknowledgment: This investigation was supported by Grant AF 61(052)–391 of the United States Air Force.

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Changes in patterns of liver enzymes in embryonic and young rats

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The mature liver has an unusually versatile metabolic capacity. Presumably the embryonic liver lacks some of these capabilities. Detailed knowledge of the changes in specific enzyme capacities should help in understanding enzymatic differentiation. Earlier studies[1] demonstrate impressive increases in flavoproteins during rapid growth. Equally marked changes in enzymes concerned with glucose and lactate metabolism have now been observed. Methods were developed of sufficient sensitivity to study a variety of enzymes in individual embryonic livers. Livers from 6 to 16 rats in each age group were analyzed for 13 enzymes. Enzyme activities, as % of the adult level, for 17 day fetal, newborn, and 9 day old rats respectively were:
Hexokinase: 398,281,188; phosphofructokinase: 307,190,137; hexosediphosphate aldolase: 229,124,105; glucose-6-phosphate dehydrogenase:–,107,63; 6-P-gluconic dehydrogenase: –,79,30; glucophosphoisomerase: –,161,91; fructose-1-P aldolase

25,78,100; glucose-6-phosphatase: 34,59,92; lactic acid dehydrogenase: 77,90,136; glycerol-P-dehydrogenase: 10,45,114; triose-P-isomerase: 71,153,106; glyceraldehyde phosphate dehydrogenase: 100, 232, 121; and phosphorylase: 50, 109 and 80.

The changes observed, are consonant with increasing capacity of the developing liver for lipid synthesis and for lactate utilization rather than formation.

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The interaction of penicillinase with a synthetic derivative of 6-aminopenicillanic acid

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Exopenicillinase formed by *Bacillus cereus* can undergo conformational changes which are not reflected in its enzymic activity[1]. It has been suggested that such changes are reversed by the substrate[2] and a system has been described where the effect of the substrate on the enzyme molecule could be demonstrated[3].

Further studies along these lines have been facilitated by the availability of non-hydrolyzable analogues of the substrate. The interaction of one of the analogues with penicillinase has been studied in some detail. It has been shown that by combining with this analogue the enzyme becomes susceptible to iodination. The effect of the analogue is competitively reversed by the substrate.

Evidence will be presented that the change in susceptibility to iodination reflects a change in the conformation of the enzyme.

The observations reported will be discussed in terms of the "induced-fit" model of enzyme-substrate interaction[4].

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Stimulatory effect of polyamines on amino acid incorporation into protein in cell-free microsomal preparations

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Spermine and spermidine were found to enhance the uptake of leucine-C^{14} by rat liver microsomes when added in suitable amounts ($(10^{-4}$M and 10^{-3}M respectively) to the conventional *in vitro* system[1].

Although the magnitude of the stimulatory effect was subject to considerable fluctuation in various experimental runs, the dose response was nevertheless remarkably reproducible within each individual experiment. The extent of stimulation varied inversely with the amount of microsomes used in the reaction. Furthermore, as shown in Table I, the effect of polyamines was critically influenced by the nature and quantity of the univalent cations included in the reaction mixture. The available data do not permit a plausible interpretation of these apparently complex interrelationships. It is clear, however, that the effect of polyamines cannot be unequivocally described unless the ionic composition of the system is rigidly defined.

The site of action of the polyamines in the present system is not yet known. It may be mentioned, however, in this connection, that specially designed experiments failed to reveal any effect of polyamines on the amino acid incorporation step into S-RNA. On the other hand it appears likely that the stimulatory phenomenon is at least partly related to the stabilizing effect of polyamines on the ribonucleoprotein particles (unpublished results, see also 2,3).

The possible physiological significance of the present findings will be discussed.

TABLE I

Influence of univalent cations on the stimulatory effect of spermidine on leucine-C^{14} incorporation into microsomal protein

cations added μmoles per ml			counts per minute per mg protein	
KCl	NH_4Cl	NaCl	control	spermidine 10^{-3}M
20	—	—	460	470
20	2.5	—	960	—
20	5.0	—	1264	—
20	10.0	—	1464	1552
50	—	—	660	1008
50	—	40	784	1024
50	—	80	400	1010
120	—	—	1080	1380
150	—	—	864	1092

The reaction mixture contained in final volume of 1 ml, the following ingedients: Tris (hydroxymethyl aminomethane), 50μmoles; $MgSO_4$, 6 μmoles; other salts as indicated in the Table; adenosine triphosphate-sodium salt, 1 μmole; guanosine triphosphate 0.1 μmoles; 3-phosphoglycerate-potassium salt, 10 μmoles; DL-leucine-C^{14} approximately 5 μC per μmole, 0.2 μmoles ($= 1.8 \times 10^6$ counts per minute in the counter used); dialyzed muscle extract (ammonium sulphate fraction 52–72% saturation) 0.4 mg protein; soluble fraction (sap), approximately 1.5 mg protein; microsomes, approximately 0.5 mg protein. Incubation at 37°C for 30 minutes.

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A cold-precipitable macroglobulin-fibrinogen complex in a case of macroglobulinemia-Waldenstrom

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Plasma, serum and protein precipitated out of the plasma at a temperature of 10° were examined by paper electrophoresis, analytic centrifugation and immuno-electrophoresis. The protein sensitive to cold precipitated in the plasma in gel form but did not precipitate in the serum. It dissolved in saline at room temperature, and precipitated again when cooled. Its amount is 1.3 to 1.6 g, which is 16–20% of the protein of the plasma and it contains 0.3–0.4 g fibrinogen.

By analytic centrifugation of the protein, four components were found. One with a 6.9 S sedimentation rate, corresponding to fibrinogen, the second with 17.7 S sedimentation rate — macroglobulin 1; the third with 24S sedimentation rate — macroglobulin 2; the fourth component had a sedimentation rate of 5.5S, lower than that of fibrinogen or γ-globulin, and higher than that of albumin.This protein may be considered an outcome of the partial dissociation of the macroglobulin.

Inpaper electrophoresis the cryo-protein complex migrates between the γ-globulin and the β-globulin fraction, in the place of fibrinogen, and when stained, shows a narrow homogenic line, characteristic of macroglobulin.

When thrombin was added to dissolved cryo-protein, a coagulate appeared which dit not appear by adding of thromboplastin and Factor V.Thereby the macroglobulin is dissociated from the fibrinogen, the fibrinogen appears in its typical coagulation form, and the macroglobulin dissolves in the serum or in saline and does not precipitate any more by cooling.

When cold coagulation took place not in the protein solution but in its gel precipitate, there appeared a typical fibrinogen coagulate and the gel dissolved.

The analysis of the cryo-protein solution, after coagulation of the fibrinogen, showed a narrow line in the common migration place. In immuno-electrophoresis there appeared a homogenic precipitation line, typical in form and place to macroglobulin β_2.

In the analytic centrifugation, the same solution showed two macroglobulin components, with sedimentation rates of 17.7S and a diffuse, very heavy component, that sedimented at the beginning ; and two light components with sedimentation rates of 3.5 S and 5.7S.

The interesting and particular feature in the cold precipitable protein is the connection between macroglobulin and fibrinogen,because only through it, the macroglobulin precipitates in cold. It may be assumed that this connection creates an aggregate of high molecular weight and causes instability, demonstrated by the effect of cooling. The cutting of this connection by turning fibrinogen into fibrin removes the instability of the macroglobulin in the solution.

The cryoglobulin effect shows that there exists between those two components, different in structure and physiological origin, an association within the plasma, which is hardly detectable in other ways. We consider it interesting to investigate not only the different protein components of the plasma, but also the connection existing between them, thus comprising the physiologic entity which we call plasma. This seems to be of interest, too, in view of the tendency to connect the influence of macroglobulins with processes of blood coagulation and its defects.

Electrophoretic and immunoelectrophoretic experiments with myelomatose sera heated to 56°C

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Much research work is being done to establish the relation between myelomatic globulin found in the serum and Bence Jones protein appearing in the urine of myeloma patients.

The appearance of Bence Jones protein in the urine can hardly be explained without assuming its presence in the serum in some form or other.

A characteristic quality of urinary Bence-Jones protein is its thermolability. In consequence we attempted to find out the effect of heating on the myelomatic fraction of the serum. For this purpose we examined 94 cases of myelomatosis. The sera were examined by electrophoresis after heating to 56° for half an hour to two hours. The effect of prolonged heating for 18 hours was examined too.

Out of 94 sera 31 coagulated by heating, most of them after prolonged heating for 18 hours. When saline was added to the coagulate the normal protein fractions dissolved and appeared on the electrophoretic band, while the abnormal fraction did not dissolve in the saline and did not appear on the electrophoretic band.

All these sera showed, upon short heating, far-reaching changes in the abnormal fraction. The myelomatic fraction in the γ-position split in two upon heating. One part retained its mobility in the electric field while the other accelerated its mobility and appeared as a new protein fraction between the γ-and β-position, or, sometimes, migrated together with the β-globulin. The original myelomatic fraction contained gluco and lipoproteins, whereas the new, separate fraction contained glucoproteins but no lipoproteins.

In the β-position, too, the myelomatic fraction split in two upon heating, and in this case one part of the abnormal fraction slowed up its mobility. Both parts contained gluco and lipoproteins.

In some of the cases in which splitting occurred, immuno-electrophoretic experiments proved the antigenic dublicity of the abnormal fraction by the appearance of a double precipitation line in the region of the abnormal fraction.

Another group is formed by 5 myelomatic sera with an abnormal fraction close to β-globulin. These do not coagulate upon prolonged heating, but upon two hours

heating the whole abnormal fraction passes the β–position and appears on the electrophoretic band together with α_2-globulin. It contains gluco-proteins and lacks lipoproteins.

These experiments seem to prove that in myelomatic serum there are proteins — sometimes apperaring together with the main abnormal fraction and sometimes apart from it — which, like urinary Bence Jones protein, are sensitive to 56 °C and lack the lipoproteinic components.

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Chromatographic fractionation of soybean proteins on diethyaminoethyl (DEAE)-cellulose and on calcium-phosphate columns

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Soy proteins (Lincoln var.) were fractionated by two different chromatographic methods: (1) on DEAE cellulose columns, *p*H 7.6; elution being performed by stepwise increase in NaCl concentration[1]; (2) on Ca-phosphate columns[2] at *p*H 6.8; elution in this case performed by stepwise increases in phosphate buffer concentration at the same *p*H. At least eight fractions were obtained by both methods. The various fractions were assayed for their inhibitory activity on trypsin, chymotrypsin and *Tribolium castaneum* larval midgut enzyme. Inhibitory activity was found in several fractions with variations in specific activities and in their ratios for the three enzymes assayed. Similar chromatographic fractionations were carried out on the *p*H 4.5 soluble (whey) and insoluble (curd) soybean proteins. A comparison between the various fractions obtained by the two different chromatographic methods will be reported.

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The synthesis of α-hydroxy-alanine

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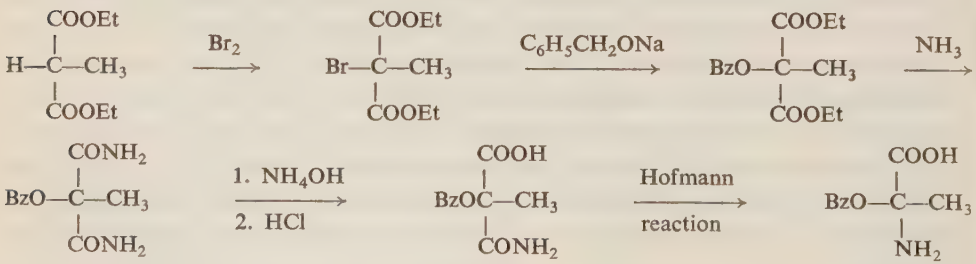
α-Hydroxy-α-amino acids, such as α-hydtoxy-alanine and α-hydroxy-valine, have been found to constitute part of the peptidic side chain of certain ergot alkaloids. Since these substances are extremely unstable, because the amino and hydroxyl functions are attached to the same carbon atom, attempts for their synthesis must ensure the reversible protection of at least one of these functions. A general method for the preparation of α-acylamino-α-substituted acids has recently been published[1], but these derivatives cannot be utilized for the synthesis of the peptidic side chain of ergotamine or ergosine, etc., because of the impossibility of removing the N-acyl groups employed without rupturing the peptide bonds.

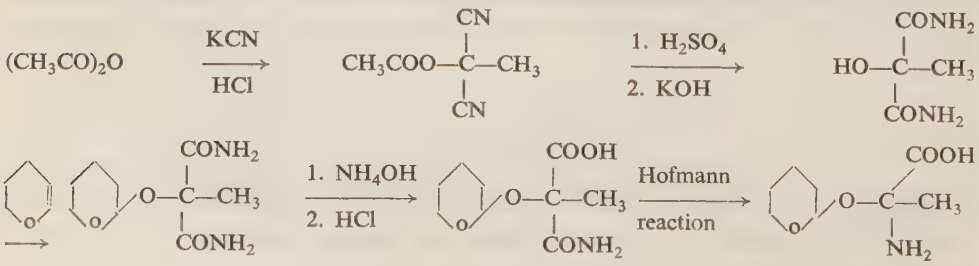
In the following scheme two routes to α-hydroxy-α-amino acids (whose hydroxyl group is protected), employed by us for the synthesis of α-hydroxy-alanine, are described. In route I the hydroxyl was protected by its benzyl ether, in route II by the tetrahydropyranyl-group. This latter group is much more suitable since it is not attacked by the alkaline conditions prevailing in the Hofmann reaction, whereas the benzyl-group is largely split off, leading consequently to the destruction of the α-hydroxy-α-amino acid formed.

Since partial hydrolysis of the tartronic ester to the semi-ester, from which the monoamide was to be formed, could not be realized, the key-reaction consisted in the treatment of the diamides with aqueous ammonia in a closed tube at 100–120°[2], a procedure by which the desired monoamides were obtained in good yields.

Route I



Route II



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Canaline derivatives and compounds related to cycloserine

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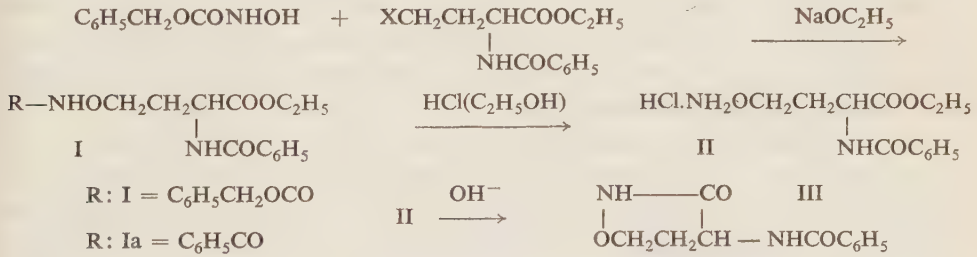
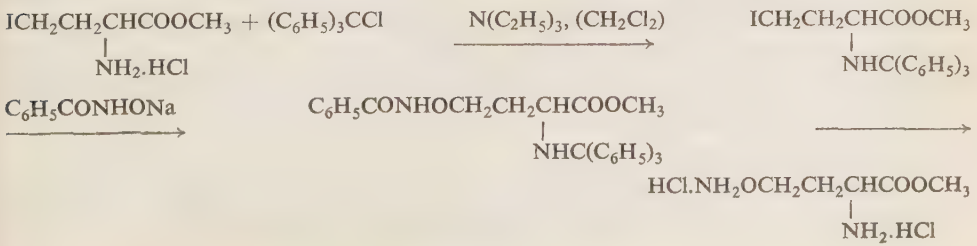
Canaline and the antibiotic cycloserine (oxamycin, seromycin), two natural aminooxy compounds, contain both an α-amino and an ω-aminooxy group, the former in the free state and the latter in an N-aminooxy-amidic cyclic form. In view of their related structure it was of interest to synthesize intermediates of canaline derivatives similar to both, and an analogous linear amide.

Carbobenzoxyhydroxamic acid was condensed with ethyl α-benzamido-γ-halogenobutyrate[1], to yield α-benzamido-γ-carbobenzoxyhydroxamobutyrate (I). Removal of the Cbzo-rest with hydrogen chloride in ethanol led to the γ-aminooxy ester hydrochloride (II), obtained also on treating ethyl α-benzamido-γ-benzhydroxamobutyrate (Ia) with ethanolic hydrogen chloride[2]. Liberation of the ester and cyclisation by the aid of Triton B or by tetramethyl-ammonium hydroxide yielded the α-N-benzoyl lactamic derivative of canaline (4-benzamido-tetrahydro-o-isooxazin-3-one) III:

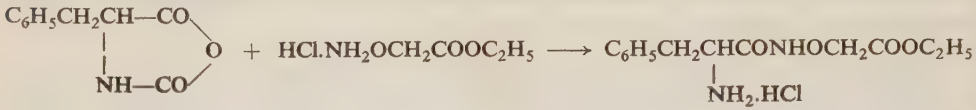


A canaline ester derivative with two protecting, acid removable groups, was prepared by tritylation of ethyl α-amino-γ-iodobutyrate hydrochloride[3] and subsequent condensation of the obtained ethyl α-tritylamino-γ-iodobutyrate (IV) with benzhydroxamic acid or carbobenzoxyhydroxamic acid:



The canaline ester dihydrochloride was also prepared by repeated cautious esterification of the hydrolysate of Ia.

Interaction between the N-carboxy acid anhydride of phenylalanine and ethyl aminooxyacetate hydrochloride led to ethyl phenylalanylaminooxyacetate hydrochloride, a free peptide of a new type between an amino and an aminooxy acid, with the amino and aminooxy groups in positions analogous to these of cycloserine:



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Oxidative activation of acyl hydrazides

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Recently, a new method was described[1] for peptide synthesis from acyl hydrazides and amines with N-bromosuccinimide (NBS). Milder oxidizing agents have now been compared to NBS in the synthesis of Cbz glycyl-glycine *p*-nitrobenzyl ester. Yields of 86% and 46% were obtained with iodine and N-bromoacetamide (NBA), respectively, compared to about 84% with NBS. N-chloroacetamide was unable to serve as a sufficiently strong oxidizing agent.

Successful synthesis was achieved if the Cbz glycyl-hydrazide and the glycine *p*-nitrobenzyl ester were present together at the instant of the addition of two equivalents of the oxidizing agent. However, a small amount (3%) of diacyl hydrazide was also found. Reaction of the acyl hydrazide and oxidant followed after a minute by addition of the amine gave as the only product which could be isolated the diacyl hydrazide. Reaction of the amine and oxidant followed by addition after a minute of the acyl hydrazide gave a small yield (10%) of the peptide and some diacyl hydrazide.

Reaction of equivalent amounts of oxidant and acyl hydrazide alone gave the diacyl hydrazide in yields of 74% (NBS), 60% (NBA) and 50% (iodine). All attempts to synthesize the peptide from authentic di(Cbz glycyl)-hydrazide, (Cbz-Gly-HN-NH-Gly-Cbz) and glycine *p*-nitrobenzyl ester with the various oxidants—failed. This indicated that a diacyl hydrazide is not an intermediate in the activation of the acyl group in the peptide synthesis.

At present the mechanism is thought to involve acyl activation by formation of a higly reactive and unstable acyl diazohalogen compound (R–C– N = N–X)

which reacts with the amine to give a peptide bond, nitrogen and hydrogen halide. In the absence of the amine and to a small extent with amine present, unreacted acyl hydrazide couples with the activated acyl group to yield the diacyl hydrazide, nitrogen and hydrogen halide.

Several peptides have been synthesized using either NBS or iodine as the oxidant. In the case of iodine oxidation the crude product is usually obtained less contaminated with side products than in the case of NBS.

This work was supported by Grant A–3083 of the National Institutes of Health, U.S.A. Public Health Service and by the National Science Foundation Grant NSF–G–13957 (to P.M.G.).

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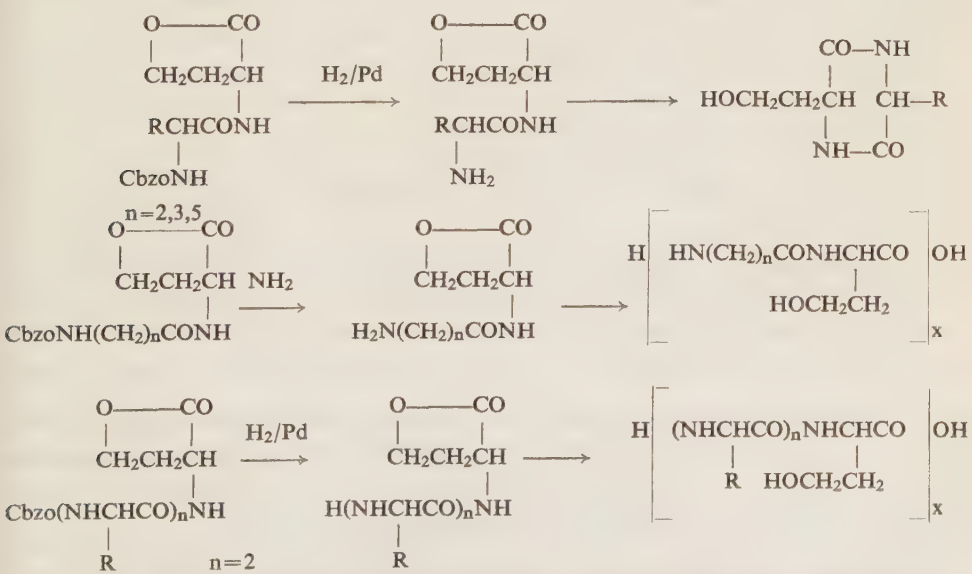
Synthesis of diketopiperazines, oligopeptides and poly-oligopeptides of homoserine by the aminolactone method

Y. Knobler, T. Sheradsky* and Max Frankel, *Department of Organic Chemistry, The Hebrew University of Jerusalem*

The aminolactone method for the preparation of homoserine peptides[1] was extended to the syntheses of mixed diketopiperazines, polydipeptides, polytripeptides and oligopeptides of homoserine.



N-Carbobenzoxypeptido-lactones[1] were set free on catalytic hydrogenation; the free peptidolactones underwent intramolecular or intermolecular amidation

in dependence on the distance of the amino group from the lactonic carbonyl group.

In peptidolactones built up by N-substitution of homoserine lactone (2-amino-4-butyrolactone) by an α-amino acid, on heating an intermolecular rearrangement took place with an opening of the lactone ring, resulting in conversion to 3-(β-hydroxyethyl)-6-alkyl-2,5-diketopiperazines (the cyclic mixed anhydrides of homoserine and the α-amino acid).

Under similar conditions, with increasing distance between the lactonic carbonyl group and the amino group, thus eliminating the possibility of ring formation, peptidolactones, derived from the aminolactone and an amino acid other than α–, underwent intermolecular amidation and formation of polydipeptides built up alternatively from the amino acid and from homoserine.

N-substitution of the homoserine lactone by an N–carbobenzoxydipeptide led on catalytie hydrogenation in neutral medium and following intermolecular amidation to the analogous poly-tripeptides, built up in sequence from the two amino acids and from homoserine.

Hydrogenation of the Cbzo-tripeptidolactone in a basic medium liberates the free tripeptide. Interaction between Cbzo–tripeptidolactone and an amino acid or a dipeptide, or between α-Cbzo-amino-γ-butyrolactone and an oligopeptide, leads to stepwise elongated oligopeptides.

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Helix formation in short poly-L-proline chains

A. Yaron and A. Berger, *Department of Biophysics, The Weizmann Institute of Science, Rehovoth*

The peptide chain of poly-L-proline can exist in two forms, I and II, to which the all cis and all trans configurations respectively have been assigned[1]. The cis helix was shown to contribute about + 300° and the trans helix about – 300° to the specific rotation of the L-prolyl residue.

In order to determine at which chain length the helix contribution becomes noticeable, a series of multichain polymers was synthesized in which poly-L-proline side chains are attached to an optically inactive core, consisting of a backbone of poly-DL-lysine bearing short polybenzyl-DL-aspartate side chains[2].

The specific optical rotations, calculated per proline residue, as a function of average chain length are given in Figure 1. The lower curve represents form I and the upper curve form II.

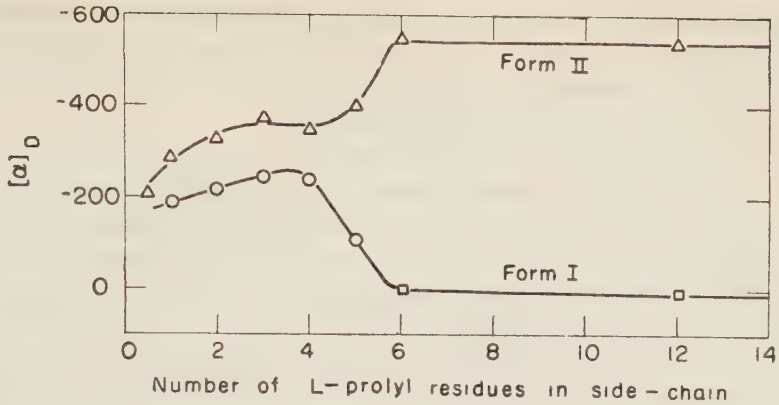

Figure 1
The specific optical rotation per proline residue of branched polymers with poly-L-proline side chains. The upper curve (form II) was obtained after complete mutarotation in the presence of formic acid. The lower curve (form I) was obtained by extrapolation of the mutarotation curves to zero time.

It can be seen that at chain length of about DP = 5 a sharp change in the optical rotation occurs in both forms. From a value of DP = 6, representing two turns of the trans helix, the rotation is independent of chain length and is identical with that of linear high molecular weight poly-L-proline. In mutarotation experiments an abrupt transition in the kinetic characteristics was observed in the same range (DP = 4 to 6).

The above results indicate that at the critical length of 6 residues a stable helical configuration is formed. As there are no hydrogen bonds present in the polyproline helices, it seems that geometrical factors involving polymer-solvent interaction are the cause for the existence of an ordered structure of the polyproline molecule.

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Water soluble multichain polyamino acids

N. Lupu, A. Yaron, M. Sela and A. Berger, *Department of Biophysics, The Weizmann Institute of Science, Rehovoth*

In the course of our work on branched polyamino acids it was of interest to obtain water soluble, non-ionized derivatives of these substances. For this purpose it was attempted to convert the benzyl ester groups of polymers containing γ-benzyl glutamate and β-benzyl aspartate residues into the corresponding hydroxyamides by means of amino alcohols.

Ethanolamine reacted with the β-benzyl esters at 100°, and within 20 min. a water soluble polymer was obtained, which however contained an appreciable amount of ionizable groups with an apparent pK of 6.5. This was interpreted as being due to the formation of weakly basic oxazoline rings[1].

When 1-propanol-3-amine was used in order to avoid this cyclization, conversion was complete at 60° within 6 hours as proved by the increase of the nitrogen content from 7% to 15% and by a quantitative chromatographic determination of the products of total hydrolysis. This analysis and the high sedimentation constant also showed that no significant cleavage of peptide bonds had occurred. The water soluble product had no ionizable groups.

Experiments with linear poly-β-benzyl aspartate and poly-γ-benzyl glutamate showed that the above reaction conditions do not cause racemization. Optical rotatory dispersion measurements were carried out with these materials in water and several organic solvents. Results of these measurements and their bearing on the problem of helix-coil transitions will be discussed.

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The cleavage of histidyl peptides by means of brominating agents*

SH. SHALTIEL AND A. PATCHORNICK, *Department of Biophysics, The Weizmann Institute of Science, Rehovoth*

Seeking a selective non-enzymatic reagent for the cleavage of peptide bonds adjacent to histidine, we have investigated the reactivity of brominating agents (such as: bromine, N-bromosuccinimide, N-bromoacetamide and N-chloroacetamide) on histidyl peptides.

(I) Cbz-L-His-GlyOH
Cbz-L-His-DL-PheOH

(II) diCbz-L-His-GlyOet
diCbz-L-His-L-LeuOMe
diCbz-L-His-DL-PheOMe (Cbz = Carbobenzoxy)

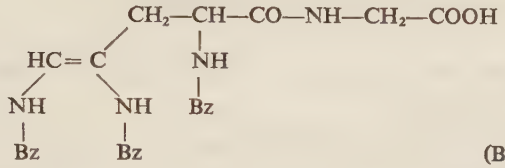
These reagents were used on the following peptides:

The reactions were performed at room temperature on dilute solutions of the peptides in acetic acid-water mixtures. The amount of the glycine, leucine, phenylalanine and their esters released was estimated by quantitative paper chromatography (Ninhydrin). The yields were 15% to 30%. Maximal cleavages were observed when one mole of peptide of the types (I) and (II) reacted with three and two moles of reagent respectively. N-Chloroacetamide did not bring about cleavage of the peptides.

* This investigation is supported by a research grant No. A-3171 from the National Institutes of Health, U.S.A. Public Health Service.

An attempt was also made to cleave histidyl peptides which had been previously modified through the Bamberger cleavage[1], i.e. the use of benzoyl chloride in mild alkaline medium at 0°C. The peptide bond in the model (III)

(III)

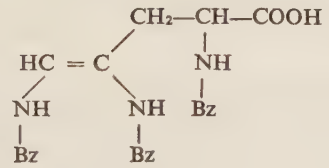


(Bz = COC_6H_5)

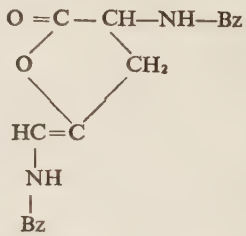
was cleaved in 20–25% yield by absorbing one mole of N-bromosuccinimide.

Using one mole of N-bromosuccinimide to react upon a Bamberger-cleaved histidine (IV), we were able to isolate a new substance, to which we ascribe the formula (V):

(IV)



(V)



Elemental analysis as well as infra-red absorption spectrum support this assumption.

The changes observed in the ultra violet spectrum during the reaction are in accordance with the assumed transformation of the acid (IV) to the lactone (V). The cleavage of the peptide bond can, therefore, be attributed to the tendency for the lactone formation.

Brominating agents are known to cleave peptide bonds adjacent to tryptophan[2] and tyrosine [3,4]. Attempts were made to employ these methods for selective cleavage of peptide bonds in proteins [5,6]. In order to investigate the possibility of cleaving peptide bonds adjacent to histidine in the presence of tyrosine and tryptophan, we tried in the first place to find out whether the cleavage near a tyrosine residue could be prevented by means of acylation of the hydroxyl group by CbzCl. We found that although the peptide (VI) was cleaved by the action of N-bromosuccinimide, the peptide (VII) was not cleaved.

(VI) N—Cbz—L—Tyr—GlyOH
(VII) N;O—diCbz—L—Tyr—GlyOEt.

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BOOK REVIEWS

HETEROMETRY, by MORDECHAI BOBTELSKY, Elsevier Publishing Co., 1960, 230 pp. 42s.

Prof. Bobtelsky summarizes in this monograph the ample work carried out by himself and his co-workers on heterometry, a new method which he invented and developed. The heterometer is an apparatus specifically designed and constructed for carrying out electrophotometric titrations involving heterogenous systems. The heterometer is a photometer with vertical light arrangements adapted to measure the light absorption of suspensions formed during titrations. A vertical glass stirrer inserted in the liquid in the reaction vessel holds the precipitate formed during the reaction in a state of suspension. On continuing dropwise titration and stirring vigorously a heterometric absorption curve is obtained. Critical points which may appear in a heterometric curve indicate the appearance of differently composed compounds. Contrary to most of the known physicochemical methods this method is not limited to the study of final reactions but also gives information on intermediate reactions.

The author, a former student of the late classical complex chemist Alfred Werner, used heterometry for examining the structure of many complex compounds and the mode of their formation. Evidence for the structure of complex salts with hydroxy acids, different thio-compounds and polyphosphoric acids was gained by Bobtelsky's heterometric studies. The author reveals the applicability of this method for various analytical purposes, especially for the determination of traces of metals in presence of foreign ions. The addition of chelating agents eliminates the influence of attendant materials on the result of heterometric titrations and makes their previous separations superfluous. These heterometric procedures are easily and quickly conducted and excel other analytical methods in sensitivity and reliability.

Bobtelsky presents in his book a very impressive amount of experimental data . Many thousands of reactions have been followed heterometrically and 50,000 heterometric titrations have been made.

The presentation of this book shows clearly the author's enthusiasm and devotion to his work. The most welcome reward for his effort will be if his book stimulates other chemists to use heterometric procedures for their research and routine work.

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CHROMOTOGRAPHIC REVIEWS. Progress in Chromotography, Electrophoresis and Related Methods. Vol. 2. Edited by MICHAEL LEDERER. Elsevier Publishing Co. 1960, Amsterdam-London-New York-Princeton.

This, the second volume of the series of annual reviews in the above field, represents a collection of six articles on various aspects of Chromatography which appeared originally during the years 1958 and 1959 in the Journal of Chromatography.

The articles are:

Review of Gas-Liquid Chromatography, by C.J. Hardy and F.H. Pollard (Bristol) (pp. 32)

Starch Electrophoresis I. Starch Block Electrophoresis, by H. Bloemendal (Amsterdam) (pp. 13).

Paper Chromatography of Dinitrophenylamino Acids, by G. Biserte, J.W. Holleman, J. Holleman-Dehove and P. Sautiere (Lille) (pp. 44).

The Chromatography of the Flavonoid Pigments, by J.B. Harborne (Hertford) (pp. 21).

The Separation of Different Types of Human Haemoglobin, by H.K. Prins (Amsterdam) (pp. 40).

Inorganic Adsorption and Precipitation Chromatography, by E. Hayek (Innsbruck) (pp. 20).

Mostly, the reviews are reprinted unchanged from the Journal of Chromatography but in some cases the undersigned found additional literature included to bring the material up to date.

As all the subjects dealt with are reviewed by specialists in their respective fields, they are competent throughout and up to date.

Again, as in the case of the first volume in this series, it can be said that this publication contains valuable, authoritative and handy information for those chemists and biochemists who do not want to bother to look up the original articles.

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